Interactions of Competing Adeno-associated Virus Type-2 and Herpes Simplex Virus Type-1 in Co-infected Cells

Dissertation

zur
Erlangung der naturwissenschaftlichen Doktorwürde
(Dr. sc. nat.)

vorgelegt der
Mathematisch-naturwissenschaftlichen Fakultät
der
Universität Zürich

von

Michael Seyffert

von

Lumnezia GR

Promotionskomitee

Prof. Dr. Cornel Fraefel (Leitung der Dissertation)

Prof. Dr. Urs Greber

Prof. Dr. Peter M. Beard

Zürich, 2014

The experimental work presented in this thesis was conducted at the Institute of Virology and the Institute of Molecular Life Sciences (IMLS) at the University of Zurich. This thesis was under supervision of Prof. Dr. Cornel Fraefel (Institute of Virology, University of Zurich), Prof. Dr. Urs Greber (IMLS, University of Zurich) and Prof. Dr. Peter M. Beard (Swiss Institute for Experimental Cancer Research (ISREC) at the École Polytechnique Fédérale de Lausanne (EPFL)).

Zürich, April 2014

Content

1. Abstract

Adeno-associated virus type-2 (AAV2) is a member of the genus *Dependovirus* and the family *Parvoviridae*. In order to enter a lytic replication cycle, AAV2 requires the simultaneous co-infection of a helper virus such as adenovirus (Ad), herpes virus or papillomavirus. In absence of a helper virus AAV2 establishes a latent infection by integrating its genome into the host cell genome, preferentially at a distinct site termed AAVS1on human chromosome 19. In the past, it has been documented that AAV2 is inhibiting replication of its helper viruses in the co-infected cell. Moreover, it has been shown that the non-structural AAV2 Rep proteins are responsible for the AAV2-mediated inhibition of helper virus replication. While the mechanism of Rep-mediated inhibition of Ad replication has been described explicitly, less is known about the mechanism how Rep inhibits herpes virus replication. In particular, although the herpes simplex virus type-1 (HSV-1), a prominent human pathogen, has experimentally been used as the preferred helper virus for AAV2 replication among the herpesviruses, to date interference of the Rep protein with the HSV-1 replication cycle has been poorly understood. Therefore, the goal of this work was to identify the mechanism of AAV2 Rep-mediated inhibition of HSV-1 replication. We demonstrate by using a set of plasmids encoding mutant and wild-type (wt) Rep that the DNA-binding and helicase domains in *cis* are necessary and sufficient for inhibition of HSV-1 replication, while the endonuclease domain is dispensable. Moreover, we show that Rep is inhibiting HSV-1 at the stage of DNA replication. Assessment of the HSV-1 protein levels revealed that Rep proteins are not diminishing HSV-1 immediate-early (IE) and early (E) gene expression which are required for HSV-1 DNA replication, but cause a clear reduction in the levels of the late (L) proteins, indicating that inhibition indeed occurs at the stage of DNA replication. The Rep activities required for inhibition of HSV-1 DNA replication precisely coincide with the activities that were required for the induction of apoptosis and DNA-damage response (DDR) in the cell. More precisely, the AAV2 Rep proteins induce a DDR in cells that includes the hyper-phosphorylation of replication protein A (RPA) which is not activated upon infection with HSV-1 alone. Therefore, we tested whether either the Rep-mediated induction of apoptosis or the activation of RPA are responsible for the Rep-mediated inhibition of HSV-1 DNA replication. However, the results of these experiments revealed that neither pathway is directly responsible for inhibition of HSV-1 replication; rather they are downstream effects resulting from the DNA-binding and helicase activities of Rep. We therefore hypothesized that the AAV2 Rep proteins can interfere with the HSV-1 DNA replication process directly. The Rep proteins may bind to putative Rep-binding sites (pRBS) on the HSV-1 genome and modify the bound DNA substrate via the helicase

activity. Of note, the Rep-binding site (RBS) is a specific nucleotide sequence motif within the AAV2 genome and the AAVS1 site required to direct the Rep proteins to the AAV2 or host DNA during replication, transcription and integration. The very same RBS nucleotide characteristics were used to identify putative (p)RBS on the HSV-1 genome. We identified nine pRBS motifs on the HSV-1 genome and demonstrated that the Rep proteins indeed are capable of binding to these sites *in silico* and *in vitro*. Moreover, we demonstrated that the helicase activity *per se* can inhibit DNA replication. The AAV2 Rep-helicase activity is inhibiting any replicating dsDNA substrate if it can bind to it, independently of the AAV2 DNA-binding domain. Our results suggest the following mechanism that contributes to Rep-mediated inhibition of HSV-1 DNA replication: the Rep proteins bind to pRBS motifs on the HSV-1 genome and modify the dsDNA via the helicase activity which is ultimately resulting in a stalled replication fork (RF). This mechanism is in analogy with the MCM2 helicase activity which is continuing to unwind the dsDNA substrate when it is uncoupled from the replication complex, e.g. during a stalled RF. This process is leading to inhibition of DNA replication, an event necessary to provide a time window for the cell to repair the damaged DNA.

In a second project, we assessed the phenotypic profile of a novel AAV2 Rep protein mutant which comprises a Tyrosin substituted for an Asparic acid at position aa-371 (D371Y). This mutation lies directly within the Rep-helicase domain and is associated with an intriguing phenotype. While the helicase activity necessary for the AAV2 DNA replication is unaffected, the helicase activity required for the inhibition of HSV-1 DNA replication is reduced. Moreover, the mutant Rep78D371Y proteins exhibit significantly less toxicity in cell culture. Specifically, the initiation of a cellular DNA-damage response (DDR) is reduced by 20-60% in cells expressing Rep78D371Y compared to cells expressing the wt Rep78. In addition, the mutant Rep78D371Y proteins are completely incapable of inducing apoptosis in transfected cells. These data led to the hypothesis that this novel AAV2 Rep mutant may augment the performance of HSV/AAV hybrid gene-therapy vectors. Essentially, in transduced cells these hybrid vectors rely on the expression of the Rep proteins to establish site-specific integration of the AAV2 ITR-flanked transgene into the host cell genome. Hence, the necessary existence of the Rep ORF on the vector in *cis* leads to the expression of the Rep proteins during production of the vector stocks and therefore the inhibition of the HSV-1 based hybrid vector DNA replication. By introducing the novel mutant Rep78D371Y ORF into the hybrid vectors, we improved hybrid vector stock titers up to 10-fold. In addition, the low toxicity of Rep78D371Y may have a positive effect on the physiology of transduced cells, diminishing safety concerns in clinical trials.

2. Zusammenfassung

Das Adeno-assoziierte Virus vom Serotyp-2 (AAV2) gehört zum Genus Dependovirus innerhalb der Familie der *Parvoviridae*. AAV2 ist ein replikations-defektes Virus und benötigt für die Replikation die gleichzeitige Anwesenheit eines sogenannten Helfervirus. Zu den Helferviren von AAV2 gehören unter anderem Adenoviren, Herpesviren und Papillomaviren. In Abwesenheit eines Helfervirus, kann AAV2 sein Genom an einer bestimmten Stelle (AAVS1) des menschlichen Chromosoms 19 integrieren. So kann AAV2 mehrere Monate bis Jahre in einer latenten Phase verbleiben bis ein potentielles Helfervirus die Zelle infiziert und die nötigen Helferproteine für die AAV2 Replikation zur Verfügung stellt. In der Vergangenheit ist gezeigt worden, dass AAV2 alle seine Helferviren an der Replikation hindern kann, möglicherweise um die Konkurrenz um zelluläre und virale Ressourcen zu minimieren. Der Mechanismus wie AAV2 die Replikation von Adenoviren inhibiert ist bekannt und wurde bereits detailliert beschrieben. Hingegen ist der Mechanismus wie AAV2 Herpesviren inhibiert nur sehr vage bekannt. Man weiss lediglich, dass der Mechanismus durch die nicht-strukturellen AAV2 Rep Proteine induziert wird. Das Ziel dieser Studie war es, diesen Mechanismus näher zu identifizieren und zu beschreiben. In einem ersten Schritt haben wir die funktionellen Domänen der Rep Proteine identifiziert, welche für die Inhibition der HSV-1 Replikation verantwortlich sind. Wir haben dazu eine Reihe von wild-typ (wt) und mutanten Rep Proteinen getestet und haben herausgefunden, dass die DNA-bindungs Domäne zusammen mit der ATPase/Helikase Domäne für die Inhibition von HSV-1 verantwortlich ist. Erstaunlicherweise ist die Endonuklease Aktivität von Rep nicht notwendig um HSV-1 zu inhibieren. Das Gen-Expressionsprofil von HSV-1 infizierten Zellen hat gezeigt, dass die Rep Proteine die Expression der HSV-1 immediate-early (IE) und early (E) Gene nicht beeinflussen, hingegen ist die Expression der late (L) Gene stark reduziert. Dies war ein erster Hinweis, dass die HSV-1 DNA Replikation selbst betroffen ist, da die Expression der L Proteinen von der HSV-1 DNA Synthese abhängig ist. Einen zweiten Hinweis haben Southern Blot Experimente ergeben, mit welchen wir die HSV-1 DNA visualisiert haben. Dabei zeigte sich, dass die Expression der Rep Gene die Produktion von HSV-1 DNA Kopien stark beeinträchtig hat.

Da die gleichen Rep Domänen, welche für die HSV-1 Inhibition benötigt werden genauso verantwortlich sind, um einen sogenannten zellulären DNA-damage response (DDR) auszulösen und die Zellen in Apoptose zu versetzen, haben wir angenommen, dass diese Effekte auf die Zelle mögliche Mechanismen sein könnten, welche für die Inhibition der HSV-1 DNA Replikation verantwortlich sind. Genau gesagt induzieren die Rep Proteine einen DDR welcher unter

anderem durch die Aktivierung der Replikations Proteine A (RPA) charakterisiert ist. Wir haben daher untersucht, ob die Aktivierung von RPA unabhängig von Rep einen Einfluss auf die HSV-1 DNA Replikation hat. Zudem haben wir getestet, ob die Rep-induzierte Inhibition von HSV-1 verhindert werden kann, wenn wir die Apoptose in Zellen gezielt unterdrücken. Beide Experimente haben jedoch gezeigt, dass keine dieser Funktionen für die Inhibition von HSV-1 verantwortlich sind. Daraus folgerten wir, dass die AAV2 Rep Proteine einen direkten Einfluss auf die HSV-1 DNA Replikation haben müssten. Die Rep Proteine könnten theoretisch mit ihrer DNA-bindungs Domäne direkt an die doppel-strängige HSV-1 DNA binden und diese mit Hilfe der Helikase Aktivität entwinden und so die DNA Replikation verunmöglichen. Die DNA-bindungs Domäne von Rep bindet nämlich an eine ganz spezifische Nukleinsäure Sequenz, welche Rep-binding site (RBS) genannt wird. Solche RBS befinden sich auf der AAV2 DNA und auf der AAVS1 Stelle im humanen Chromosom 19. Rep Proteine binden gezielt an diese DNA Abschnitte um die AAV2 Replikation, Transkription, oder die Integration zu steuern. Wir haben daher das HSV-1 Genom auf das Vorhandensein solcher RBS untersucht und haben neun mutmassliche RBS gefunden. *In vitro* Versuche haben ergeben, dass aufgereinigte Rep Proteine tatsächlich an diese RBS Stellen auf der HSV-1 DNA binden können. In einem weiteren Versuch haben wir gezeigt, dass die Helikase Aktivität von Rep unabhängig von der DNA-bindungs Domäne jedes DNA Substrat so manipuliert, dass eine Replikation nicht mehr möglich war. Mit diesen beiden Erkenntnissen konnten wir die Theorie bestätigen dass die Rep Proteine an die HSV-1 DNA binden und mit der Helikase Aktivität die Replikation inhibieren können. Diese Hypothese erhält zusätzliche Unterstützung aus der Tatsache, dass die Zelle einen sehr ähnlichen Mechanismus benützt, um die eigene DNA Replikation zu kontrollieren. Wenn die replizierenden Polymerasen einen defekten DNA Strang vorfinden, löst sich der mini-chromosome maintenance 2-7 (MCM2-7) Protein Komplex von den Polymerasen ab und entwindet einen Teil der darauf folgenden doppel-strängigen DNA. Dies hat zur Folge, dass aktivierte RPA Proteine an diese entwundene DNA rekrutiert werden und eine DDR auslösen, welche den Zell Zyklus stoppt damit die Zelle Zeit gewinnt um die beschädigte DNA zu reparieren. Solche so genannten blockierten Replikationsgabeln könnten sich so ähnlich auf der HSV-1 DNA bilden, wenn die Rep Proteine dort binden und die DNA entwinden aber keine Replikation stattfindet.

In einem zweiten Projekt haben wir den Phänotyp von Rep Proteinen untersucht, welche eine neu entdeckte Mutation aufweisen. Diese Mutation betrifft eine Aspartansäure an der Aminosäure Position 371, welche mit einem Tyrosin substituiert ist (D371Y). Diese Mutation liegt genau zwischen den Walker Motiven A und B der Helikase Domäne von Rep und weist einen

interessanten Phänotyp auf. Die Rep Proteine mit dieser Mutation sind einerseits immer noch in der Lage die AAV2 Replikation, welche ganz von der Helikase Aktivität abhängig ist zu steuern. Andererseits sind diese mutierten Rep Proteine nicht mehr in der Lage die HSV-1 Replikation zu inhibieren. Zusätzlich haben wir gezeigt, dass diese mutierten Rep Proteine auch nicht mehr in der Lage sind eine zelluläre DDR oder Apoptose auszulösen. Diese Beobachtungen könnten für die Konstruktion von HSV/AAV Hybrid Gentherapie Vektoren von grosser Bedeutung sein. HSV/AAV Hybridvektoren enthalten unter anderem das AAV2 Rep Gen um die Integration des Transgens ins Wirtszellgenom zu ermöglichen, damit eine möglichst lang anhaltender therapeutischer Effekt erzielt werden kann. Da jedoch die Rep Proteine die HSV-1 DNA Replikation inhibieren und einen toxischen Effekt in der Zelle auslösen, sind diese Vektoren nicht sehr vielversprechend. Die Herstellung von Hybridvektoren ist abhängig von der HSV-1 Replikation und wird somit von den Vektor-kodierten Rep Proteinen unterdrückt, was zur Folge hat, dass die Produktion sehr ineffizient ist. Zudem wäre die Toxizität der Rep Proteine nicht kompatibel mit einer Verwendeung der Vektoren in klinischen Versuchen. Wir haben deshalb das wt Rep Gen in den HSV/AAV Hybrid Vektoren mit dem mutanten RepD371Y Gen ersetzt. Die daraus entstandenen Vektor Präparate sind tatsächlich bis zu 10-mal höher konzentriert als herkömmliche Hybrid Vektor Präparate. Die höheren Vektor-Titer und die stark reduzierte Toxizität führen zu einer verbesserten Effektivität der HSV/AAV Hybrid Vektoren.

3. Introduction

3.1. Background

When almost fifty years ago M. David Hoggan discovered the human adeno-associated virus (AAV) in contaminated adenovirus preparations by electron microscopy (EM), he and his coworkers had no idea that their discovery would result in the emergence of the first licensed viral gene therapy product in Europe, named Glybera (53). Basically, Glybera (alipogene tiparvovec) is a recombinant AAV (rAAV) consisting of an AAV1 capsid enclosing the transgene encoding lipoprotein lipase (LPL), which is flanked by AAV2 inverted terminal repeats (ITRs). This vector can be administered intramuscularly and is to be considered as a safe and effective treatment of LPL deficiency (15, 18, 157). This amazing development of AAV from a negligible contaminant to the most promising gene therapy vector platform nowadays is not self-evident. Since its discovery, scientists all over the world contributed piece by piece to the knowledge we have today, necessary to develop Glybera.

Shortly after its discovery it became clear that AAVs are nonpathogenic, rather it was found that AAVs showed a tendency to inhibit tumorigenicity of other viruses such as adenoviruses, papillomaviruses or herpes viruses. Despite its innocuousness, AAV gained immense scientific popularity. So also in the lab of Kenneth I. Berns, who first described the AAV life cycle including its property to establish a latent infection by integrating its genome in a well-defined locus within the human chromosome 19, termed AAVS1 (10, 11, 25, 65–68, 114). Driven by pure curiosity and fascination about this novel virus, a number of laboratories designated their work in investigating this intriguing virus. The switch from basic AAV research to AAV vector development and gene therapy applications was made by the laboratories from Muzyczka and Carter in the early 1980s, when they succeeded to clone the AAV DNA, which facilitated the engineering of rAAV vectors (73, 112, 113, 135). This achievement is considered the beginning of a new era of AAV research, where AAVs were studied intensively with the aim to use AAV one day as a gene therapy vector. This was the base of most of the knowledge about AAV we have today.

3.2. Adeno-associated Virus Type-2 (AAV2)

AAV classification and biology

The family of the *Parvoviridae* can be divided into two subfamilies: the *Densovirinae* and the *Parvovirinae* according to their prevalence in insects and vertebrates respectively. The vertebrate parvoviruses itself are classified into two functional groups, which are the autonomous parvoviruses and the dependoviruses, which differ fundamentally in their biological life cycle (9). The dependoviruses comprise the genus *Dependovirus* and include the species adeno-associated virus (AAV), which are unique among DNA viruses as these viruses are in contrast to the autonomous parvoviruses dependent on a co-infection with a so called helper virus to enter a lytic replication cycle. Ironically, helper viruses for AAV are the very same viruses AAV is inhibiting in co-infected cells, namely adenoviruses (Ad), papillomaviruses or herpes viruses (16, 53, 141, 150). In absence of a helper virus however, AAV undergoes a latent infection by integrating its genome into a well-defined site in the human chromosome 19 at a locus termed AAVS1 (66, 114, 131, 152). While the mechanism how AAV integrates its DNA into the host genome is poorly understood, the replication of AAV has been studied intensively and is well known. Among the more than 40 AAV serotypes, which include primate, avian, bovine, canine, equine and ovine serotypes, the human AAV serotype 2 (AAV2) is the most studied and best known dependovirus to date.

AAV2 structure and genome organization

The AAV2 particle is a small, non-enveloped, icosahedral capsid, built up by three structural proteins termed VP1, VP2 and VP3. The capsid consists of about 60 protein subunits of which 90% is made up by VP3. The size of AAV2 capsids vary between 20 and 25 nm and enclose a ssDNA genome of about 4.7kb in size (75). The whole genome comprises only two open reading frames (ORFs) *rep* and *cap*, which are flanked by two inverted terminal repeats (ITRs) of about 145nt (Fig. 3.1). The *rep* ORF is regulated by two promoters (*p5* and *p19*) which together with a common splicing site encode four Rep proteins termed Rep40, Rep52, Rep68 and Rep78, named according to their apparent molecular weight (23) (Fig. 3.1). The two major Rep proteins Rep68 and Rep78 are required for AAV2 DNA replication, transcription, site-specific integration and for inhibition of helper virus replication, whereas the smaller Rep40 and Rep52 are believed to be required for packaging and transcription (23, 86). The *cap* ORF is regulated by the *p40* promoter. All three capsid proteins are translated from two alternatively spliced mRNA species, which are 2.3 and 2.6 kb in size

respectively. The smaller mRNA species is usually the preferred one, where the AUG codon from which the synthesis of VP1 starts is cut out, leading to the overall reduced levels of VP1. From the next AUG codon within this mRNA species the VP3 protein is synthesized. In the same reading frame, upstream of that codon is an ACG start codon located, which is responsible for synthesis of VP2 (6, 7, 20, 91, 136) (Fig. 3.1). In addition, it has been reported recently that a nested ORF within the *cap* gene encodes a protein designated assembly-activating protein (AAP), which is believed to be required for AAV2 capsid assembly in the nucleolus (95, 122).

The AAV2 life cycle and DNA replication

Attachment and entry of the virion into the cell is mediated by heparan sulfate proteoglycans (HSPG), the primary cell surface receptors (130). In addition, fibroblast growth factor receptor-1, integrin alpha-V-beta-5 and hepatocyte growth factor receptor act as co-receptors (61, 104, 129). Adsorption of the particle at the cell membrane is induced by dynamin-dependent endocytosis via a clathrin-coated pit. After AAV2 has escaped the endosomal pathway, the capsids are organized in a peri-nuclear pattern, before the genome is internalized within the nucleus (5, 31, 46, 115, 151, 156). Once the AAV2 DNA has reached the nucleus, low level DNA replication occurs from the ssDNA template to initiate expression of the major Rep proteins 68 and 78, which are necessary for repressing all AAV2 promoters and initiating integration in the absence of a helper virus (68, 76, 114, 131). After integration of the AAV2 genome within the host genome, the virus can remain latent during several numbers of cell cycles. Upon infection with a helper virus the latent AAV2 genome is rescued and synthesis of all AAV2 proteins is induced (113, 145, 147). In the case of Ad as a helper virus, the Ad E1A protein is trans-activating transcription from the p5 promoter and initiates synthesis of Rep68 and Rep78 (21, 72, 106). At the same time, the Ad E1A protein induces S-phase, where all cellular replication proteins necessary for AAV2 replication are produced (92). In the case of herpes virus as a helper virus (e.g. herpes simplex virus type-1 (HSV-1)) the ICP0 protein is transactivating the p5 promoter and inducing synthesis of the major AAV2 Rep proteins (39). A detailed summary of helper factors from HSV-1 and the corresponding mechanisms are discussed in section 3.4 (Helper factors provided by HSV-1 for AAV2 replication).

AAV2 DNA synthesis is very unique among DNA viruses as it consists of a few remarkable features. Palindromic sequences at both ends of the genome facilitate folding of the ITRs into a T-shaped hairpin structure (Fig. 3.2 A). The ITR hairpins can appear in two

orientations, flip and flop, due to inversions within the first 125 nucleotides. Both ends consist of a Rep-binding site (RBS), which is characterized by three GAGC repeats, and is followed by a terminal resolution site (TRS) (109) (Fig. 3.2 A). The hairpin structure at the 3' end of the ssDNA genome creates a primer for Rep-mediated DNA replication and is essential to convert the single-stranded viral DNA into a double-stranded template for transcription and replication. The two sequence elements RBS and TRS act as a minimal origin of DNA replication and are equally essential for AAV2 DNA replication (143, 146). Second strand synthesis follows a unique leading-strand synthesis mechanism and is supported by either the cellular or the helper virus replication machinery (36, 58, 93, 98, 99, 149). After second-strand synthesis, binding of the major Rep proteins at the RBS induces a site specific nick at the TRS, allowing the 3' ITR to unfold in order to complete the replication process (93). The resulting complete dsDNA template is termed monomer extended form (mE). The following process, termed re-initiation, where either of the DNA ends can fold into new hairpins, leads to leading-strand displacement and creates a new primer for the next round of replication. This newly synthesized ssDNA template for replication is termed monomer turnaround form (mT). The simultaneously generated single-stranded genome is readily used for packaging. However, if re-initiation occurs before the hairpin structure of the mT form is resolved by terminal resolution the continuing DNA synthesis generates a double-stranded dimer in which two genomes are covalently linked by a single ITR. These structures can occur in two conformations: head-to-head or tail-to-tail and are termed dimer turnaround (dT) or dimer extended (dE) form (54, 144). The complete AAV2 DNA replication process is summarized in Figure 3.2 B.

This unique system of DNA replication is often called "the rolling hairpin model" in analogy to the rolling-circle model, which other DNA viruses such as HSV-1 utilizes (132).

The AAV2 Rep proteins: structure and function

The AAV2 Rep ORF is under control of two different promoters *p5* and *p19*. Together with a common splicing site, it is encoding four different Rep proteins, which differ in their size and therefore also in their composition of structural and functional domains (Fig 3.3). The complete Rep ORF is consisting of a set of distinct elements of which the most important are discussed below and summarized in Figure 3.3.

At the very N-terminal region, the combined DNA-binding and endonuclease domains are located (27, 51, 52). The DNA-binding domain (map position 1-200) is responsible for binding to dsDNA templates at specific RBS motifs. The minimal RBS motif is the consensus

sequence GAGYGAGC (26). Within the DNA-binding domain lie two rolling circle replication (RCR) motifs termed RCR2 and RCR3 (51, 120). These two motifs are essential for the terminal resolution process during DNA replication (120) and for genome integration (137). Only the major Rep proteins Rep68 and Rep78 contain a DNA-binding and endonuclease domain, because the p19 promoter responsible for the expression of Rep40 and Rep52 is located right after the DNA-binding motif at map position 225. The helicase domain, which claims 40% of the whole ORF (map position 225-490), is located right after the DNA-binding and endonuclease domain. The AAV2 Rep helicase belongs to the superfamily 3 (SF3) helicases, which are encoded mainly by small DNA viruses (57). The complete helicase domain can be subdivided into two main components and several subcomponents. First, from map position 225 to 278 an α-helix domain responsible for hexamerization is located. The rest of the domain is consisting of a typical AAA+ domain (50, 80). This section can be subdivided into different components. The ATPase domain (map position 329-490) (80) is consisting of the typical Walker motifs (50). In SF3 helicases the Walker motifs A, B and B' are separated by short uniform sequences, but unlike other superfamily helicases, the SF3 helicase consists of a third Walker motif C, which is located between the Walker motif B and the rest of the C-terminal protein domain (45). Typically, SF3 helicases form hexameric rings and rely on a DNA-binding domain in order to bind to DNA templates. The Rep helicases have been shown to bind either RBS motifs via the N-terminal DNA-binding domain, or non-specifically via the helicase domain (160–162). At the C-terminal end of the protein, the PKA binding site is located, which is consisting of a Zn-finger motif (55, 116) and a PKI like motif (29). While the Zn-finger motif is responsible for inhibition of the cell cycle, the PKI like motif is responsible for inhibition of Ad DNA replication.

All these functional domains are required for the vital processes during the AAV2 life-cycle and are regulated in a very tight fashion in order to keep the sensitive balance of self-replication and inhibiting helper virus replication while maintaining the cellular homeostasis. In addition, the capability of mediating site-specific integration makes the Rep proteins a complete survival kit, which is one of the prominent characteristics of this multifunctional protein. Here, we discuss a selection of vital functions the Rep proteins are performing during the AAV2 life-cycle:

Replication

As described above, the Rep proteins play an essential role during the DNA replication process by introducing site-specific nicks at the TRS and providing helicase activity to

unwind the hairpin termini during resolution (59, 100). The terminal resolution process can be conducted exclusively by the AAV2 Rep proteins and no other cellular or helper virus derived factor is capable of replacing or mimicking this unique Rep activity. During terminal resolution, the large Rep proteins (68 and 78) bind to the DNA-binding motif consisting of three GAGC tetra-nucleotide repeats (GAGCGAGCGAGC) located at nucleotide position 15 on the 5' ITR (84, 85). The subsequent nicking activity is performed at the TRS by the Rep endonuclease activity, which results in a gap on the original parental strand. Rep covalently binds to the 5`-terminus of this gap and the newly created 3`-terminus serves as the primer for repair synthesis of the remaining ITR sequence (13). Thereby, the ATP-dependent Rep helicase activity is unwinding the hairpin structure of the ITR, allowing the cellular or helper viral DNA polymerase to complete replication (35). Of note, Rep proteins are multimeric enzymes, which can form typical pentameric or hexameric rings, although the true oligomeric nature is unclear (162).

Integration

AAV2 latency is accomplished by either episomal maintenance of the genome in the nucleus, or by site-specific integration at the AAVS1 locus within chromosome 19 (32, 148). The integration process is mediated by the two major Rep proteins Rep68 and Rep78. Both can function as a bridge to link the AAV2 genome to the pre-initiation site at the AAVS1 by binding either RBS elements. The resulting close proximity of the two DNA strands is believed to facilitate a non-homologous deletion-insertion recombination event leading to the integration of the complete AAV2 genome (40, 76, 77, 138, 152, 158, 159). The AAV2 integration site AAVS1 on the human chromosome 19 is closely linked to the genes TNNT1 and TNNT3, which both encode muscular troponin T isoforms (33). Because the integration process leads to chromosomal rearrangements at that site, it would be conceivable that the expression patterns of these two genes are affected. However, none of those two genes show altered expression, which is due to a partial duplication process occurring during Rep-mediated integration (49). In addition, chromosomal rearrangements would not affect the multi-nuclei muscle cells to the same extend as cells with only a single nucleus, since unaffected alleles within the same cell could compensate for the affected allele. However, the complete mechanism how AAV2 integration occurs exactly is still unclear.

Transcription

The p5 promoter contains a Rep-binding site (RBS) consisting of two GAGC repeats, which allows direct binding of Rep proteins via their DNA-binding domain (100), however with a

slightly reduced binding affinity compared to the RBS located on the ITR (41). Together with the RBS located on the ITR, they are involved in the Rep-mediated regulation of transcription from all AAV2 ORFs (56, 70, 101). For instance, in presence of a helper virus, the two large Rep proteins 68 and 78 are repressing the p5 promoter, while the two small Rep proteins 40 and 52 are capable of de-repressing the promoter, most probably by interaction with the large Rep proteins (69, 71, 101). This mechanism allows the virus to tightly control the ratio of DNA replication, gene expression and DNA packaging during the lytic life cycle.

Packaging

AAV2 capsid assembly and DNA packaging occur in the nucleolus of infected cells (155). The two small Rep proteins Rep 40 and Rep 52 are required for the helicase-mediated packaging of ssDNA genomes into preformed AAV2 capsids. J.A. King proposed a model, where capsid immobilized Rep proteins function as a molecular motor pump to translocate the DNA molecules directly into the capsids (63, 119). They could demonstrate that the site-directed translocation process occurs from the 3'-end of the genome, which is in correlation with the 3'-5' processivity determined for the Rep40/52 helicases (50).

Inhibition of helper viruses

Inhibition of helper virus replication in the co-infected cell is of great interest for AAV2, since an additional replicating virus in the cell most probably would compete for cellular resources and could affect the cell-cycle in disadvantage for AAV2 replication. Therefore, AAV2 has evolved distinct pathways to inhibit helper virus replication. The intriguing part of this achievement is the strategies AAV2 has developed, where indeed helper virus replication is inhibited, but at the same time helper factor supply from these viruses is not affected. AAV2-mediated inhibition of Ad replication has been among the best studied examples, whereas inhibition of other helper viruses, such as herpes viruses or papillomaviruses is less well understood. Interestingly, as different the life-cycle and cell tropism of the helper viruses, as versatile are the strategies which AAV2 has developed for inhibition. However, one factor all strategies have in common: they all are driven and mediated by the AAV2 Rep proteins.

It has been shown that the AAV2 Rep proteins are capable of binding to the Ad E2A promoter directly, independently of a consensus RBS located at this site (19). Also, Rep is known to bind to a 55-bp DNA fragment within the Ad major late transcription promoter (MLP) via interaction with the cellular TATA box-binding protein (TBP) (96). These interactions of Rep with the Ad genome are mediating inhibition of transcription and therefore

might directly affect Ad replication. However, although the inhibition of these two promoters leads to decreased levels of E2A and E4 expression, the reduced levels of those proteins seemed not to be solely responsible for inhibition of Ad DNA replication. Rather, the AAV2 Rep proteins might inhibit Ad DNA replication directly (134). In addition, AAV2 also is restricting Ad replication via the inhibition of the cAMP-dependent protein kinase A (PKA) (29). Ad utilizes the PKA/CREB pathway to induce the switch from the early to the late replication stage by inducing DNA replication and late gene expression. The PKI like motif on the C-terminal region of Rep however is inhibiting PKA activity in the co-infected cell, thereby indirectly inhibiting Ad DNA replication.

In the case of herpes virus as the helper virus, the mechanism how the AAV2 Rep protein is inhibiting replication is not so clear. So far, it has been shown that AAV2 inhibits HSV-1 DNA replication in simian virus 40 (SV40)-transformed hamster cells (4). Also, the same group determined in a follow up study that expression of the AAV2 Rep gene only was responsible for the inhibition of DNA replication initiated by the HSV-1 oriS (47). Another study by Kleinschmidt et.al determined the sequence elements of Rep required for inhibition of HSV-1 induced DNA replication. They showed that specific sequence elements at the N-terminus of Rep are responsible for this inhibition and that Rep proteins consisting of 60% of their internal regions are still capable of inhibiting HSV-1 replication (64). These observations are in agreement with other studies, which were assessing HSV/AAV hybrid vectors and their capability for site-specific integration (48, 142). They observed that hybrid-amplicon vector titers were significantly reduced when the *rep* gene is provided either in *cis* within the same vector or in *trans* by co-transfecting a Rep expressing plasmid (48). A more recent study by Glauser et al, which investigated competing AAV2 and HSV-1 DNA replication in the same cell, demonstrated that wtAAV2, but not *rep*-deficient rAAV2, is inhibiting formation of HSV-1 replication compartments. Moreover, that study showed that Rep-mediated inhibition of HSV-1 DNA replication is dose dependent and leads to strongly reduced expression levels of the HSV-1 late genes including VP16 and glycoprotein C (gC) (43). The latter observation provides further evidence that Rep-mediated inhibition of HSV-1 replication occurs at the stage of HSV-1 DNA replication.

However, the exact mechanism how the AAV2 Rep proteins are inhibiting HSV-1 DNA replication still is not clear and needs to be elucidated.

3.3. Herpes Simplex Virus Type-1 (HSV-1)

HSV-1 classification and biology

Herpes viruses belong to the family of the *Herpesviridae*, which can be divided into three subfamilies according to their host range and their life-cycle: the *Alphaherpesvirinae*, the *Betaherpesvirinae* and the *Gammaherpesvirinae* (8). All herpes viruses share the ability to follow either a lytic or a latent infection. To date, there are eight human herpes viruses identified. The most common human herpes virus is HSV-1, which belongs to the subfamily of the *Alphaherpesvirinae*. HSV-1 is causing mucosal eruptions at the site of infections, which can reoccur at the same site upon reactivation from latency, because latency usually is established in sensory neurons of the trigeminal ganglia, which are innervating the mucosal tissue (24, 107, 126). Typically, HSV-1 is infecting the mucosal tissue in and around the oral region and causing characteristic vesicular blisters, which can disrupt and therefore mediate shedding of newly synthesized virus. Seroprevalence in the human population is relatively high at around 60 to 90% (22).

HSV-1 structure and genome organization

The HSV-1 virion is built up by three structural components which are the capsid, the tegument and the surrounding envelope (35) (Fig. 3.4). The capsid is enclosing a 152 kb dsDNA genome, which encodes approximately 85 genes. The surrounding tegument layer comprises a distinct set of tegument proteins, which are involved in e.g. viral capsid trafficking from the cell membrane to the nucleus (30, 83, 121), modulation of host cell gene expression (79) and onset of viral gene expression (62). The most outer envelope layer is derived from the host cell and consists of viral glycoproteins required for receptor binding, cell to cell spread, and entry (2, 3, 14, 103).

The viral genome is a linear double stranded DNA molecule and has a unique structure. It is divided into two covalently joined segments, unique long (U_L) and unique short (U_S), which are flanked by inverted repeat regions (TR_L, IR_L, IR_S and TR_S), where the IR sequences are linking the two unique segments (Fig. 3.5). The genome contains three origins of DNA replication (*ori*), which are located within the U_L (*ori*L) and within the TR_S (*ori*S), respectively (127). DNA packaging/cleavage signals (*pac*) are located at each end of the genome and within each IR sequence (28).

HSV-1 life cycle and DNA replication

After binding and stabilization of the HSV-1 virion at the cell membrane mediated by the viral glycoproteins, the capsid is released into the cell (105, 118). The main HSV-1 receptors on the cell surface are heparan sulfate proteoglycans (118), but attachment and entry is supported by several co-receptors, the so called herpes virus entry mediator protein receptors (Hve) (102, 105). After entry of the capsids into the cytoplasm, they migrate along microtubules to the cell nucleus (121), where the genome is released directly into the host cell chromatin and circularizes. After VP16-mediated transactivation of specific viral genes, which are encoding for transcription factors, the lytic replication cycle of HSV-1 is initiated. Gene expression occurs in a temporally regulated cascade; immediate early (IE), early (E) and late (L) (35, 108). IE proteins mainly have regulatory functions and initiate expression of the E genes. The E proteins comprise enzymes necessary for viral DNA replication, including the viral polymerase (UL30), and are therefore required for the expression of the L genes, as these genes are relying on the onset of DNA replication. Most of the late genes are encoding structural components, which consist of the capsid proteins and the envelope glycoproteins.

HSV-1 DNA replication follows a yet not fully understood rolling-circle replication mechanism. Replication is initiated at either oriL or oriS and is mainly mediated by the viral replication complex formed by the helicase-primase complex (UL5, UL8 and UL52) and the viral polymerase (UL30 and UL42). The ssDNA binding protein ICP8 (UL29) and the origin binding protein (UL9) stabilize the replication machinery and are essential replication factors (78, 81). All viral replication events are located in the nucleus within distinct areas termed replication compartments (RCs) (123). Those structures arise at 1-2 hours after infection and start as punctuate patterns associated with ND10 bodies, which constantly grow during the course of DNA replication until they engage the complete nucleus (17, 82).

3.4. Interaction between HSV-1 and AAV2

When it was discovered that herpes viruses provide helper factors for AAV2 replication, it was not clear, which proteins are involved and to what extend helper factors are supporting AAV2 replication. It was believed that herpes viruses are only partial helper viruses (12). A study by Buller and coworkers then demonstrated that HSV-1 and HSV-2 are complete helper viruses for AAV2 replication (16). Interestingly, not only alphaherpesviruses can

provide helper functions, but also herpes viruses of the subfamily of the betaherpesviruses, such as the human cytomegalovirus (HCMV) (87) and human herpes virus 6 (HHV6) can provide full helper functions for AAV2 replication (133). The best characterized herpes virus as a helper virus for AAV2 however is HSV-1.

Helper factors provided by HSV-1 for AAV2 replication

The first study, which was determining the minimal set of helper factors from HSV-1, was published by Mishra and Rose, who found that at least five of seven HSV-1 genes necessary for HSV-1 DNA replication (UL5, UL8, UL9, UL29 and UL30) are necessary to support AAV2 DNA replication (88). They stated that none of these helper factors are strictly required for AAV2 replication; however, they demonstrated that the transcriptional regulatory protein ICP4 was required, most probably to trans-activate HSV-1 helper genes. A second study by Weindler and Heilbronn suggested that only four factors are required for AAV2 DNA replication, in particular UL5, UL8 and UL52 encoding the helicase-primase complex and the ssDNA-binding protein UL29 (ICP8) (149). However, it has also been shown later that even a mutant helicase-primase complex could still provide helper functions, which was somehow unexpected (128). All these observations together suggest that the UL29 (ICP8) gene is the key helper factor for AAV2 DNA replication. This notion is further confirmed by the fact that ICP8 is strongly co-localizing with AAV2 Rep proteins in AAV2 RCs, whereas ICP4 is not (43, 128). These data confirm that ICP4 is not a required helper factor for AAV2 DNA replication. In addition, the role of the HSV-1 ICP0 protein for AAV2 replication was investigated (88). It turned out, that ICP0 was not directly involved in DNA replication, rather it transactivates the p5 promoter resulting in the onset of Rep expression from latent AAV2 genomes (38, 39). A more recent study by Nicholas et al. revealed another factor, UL12 that enhanced AAV2 replication (97). The UL12 protein was co-localizing with AAV2 RCs, but the function of UL12 during AAV2 replication remains unclear.

Overall, the nature of helper functions provided by HSV-1 differs completely from the helper functions provided by Ad. While Ad is contributing on the level of cell cycle manipulation and transactivation of gene expression, HSV-1 is rather supporting AAV2 DNA replication directly. However, recently it became clear that the cell cycle also seems to be an important factor for Rep-expression and AAV2 replication (1, 139, 140).

Interaction between HSV-1, AAV2 and the cell

A recent study by Vogel and colleagues has demonstrated that AAV2 modulates the host DNA damage response (DDR) induced by HSV-1 in the co-infected cell and therefore may modulate the fate of the cell cycle (140). Generally, HSV-1 is inducing a DDR in infected cells characterized by the activation of the MRN complex, the phosphatidylinositol-3-kinase-like kinase (PIKK) ataxia-telangiectasia mutated (ATM), p53 and Rad51 (34, 74, 117, 153), but not the ATM- and Rad3-related kinase (ATR) (89, 90, 154). However, it has become clear that HSV-1 / AAV2 co-infection leads to phosphorylation of ATR, but the ATR- pathway appeared not to be activated, as downstream targets such as the checkpoint kinase 1 (Chk1) were not phosphorylated (140). A second observation revealed that the HSV-1 ICP0-mediated degradation of DNA-PK_{CS} is significantly delayed during co-infection, which is leading to the activation of downstream targets such as p53, replication protein A (RPA) and the checkpoint kinase 2 (Chk2) (140). The DNA-PK dependent activation of p53 and Chk2 in co-infected cells, which are naturally phosphorylated by ATM, was confirmed in ATM-deficient cells, where p53 and Chk2 were still activated most probably by DNA-PK_{CS} (140).

However, whether these changes in DNA damage signaling are indeed promoting the cell cycle in favor of AAV2 DNA replication and what factors from AAV2 are required still needs to be elucidated.

A more global approach was performed by two studies which identified cellular components contributing to AAV2 replication in HSV-1 co-infected cells (94, 97). They identified a large set of cellular and viral proteins associated with either the AAV2 Rep or RCs or both. A summary of the results from these studies are reviewed in (139).

Interaction between HSV-1 and AAV2 Rep

Specific interactions between the AAV2 Rep proteins and HSV-1 replication are poorly investigated. As summarized in section 3.2 (Inhibition of helper viruses), it is known that the AAV2 Rep proteins are responsible for the inhibition of HSV-1 DNA replication, but the exact mechanism is not known. The discovery of this process would be of great interest, as it would provide critical knowledge that helps to understand the complex interactions of those two viruses in the co-infected cell. An interaction model of two different viruses infecting the same cell forms the basis for understanding even more complex interaction models in which more than two pathogens co-exist in an organism, a tissue or a cell, which is a normal state in higher organisms.

3.5. HSV/AAV Hybrid Vectors

The potential of AAV as a gene therapy vector has been discovered almost thirty years ago. Since then, AAV has been employed successfully to develop gene therapy vectors such as the recently emerged gene therapy product Glybera (15, 18, 157). The success of AAV vectors is based on various advantages of this virus (44): (i) until now, AAV has not been associated with any human or animal disease; (ii) without a helper virus, wtAAV can integrate its genome at a specific site on the human chromosome 19 (AAVS1) and therefore has the potential to deliver transgenes directly to the host genome assuring long-term transgene expression on a physiological level; (iii) AAV can infect many different dividing and non-dividing cell types; (iv) the AAV genome is organized rather simple and is therefore easy to clone and manipulate. However, a number of drawbacks limit the use of AAV as a gene therapy vector dramatically. The relatively small transgene capacity of about 4.7kb and the fact that the toxic Rep proteins are required for integration are two of the most important limitations AAV vectors have.

HSV-1 derived gene therapy vectors are particularly promising for treating disorders of the nervous system. Several features make HSV-1 suitable as a gene delivery vector: (i) more than 20% of the HSV-1 genes are non-essential for replication in cell culture and can be replaced with transgenes of interest; (ii) similar to AAV, HSV-1 can infect both dividing and non-dividing cells; (iii) the episomal HSV-1 genome can enter a state of latency in neurons, however with reduced transcriptional activity. Two fundamentally different HSV-1 vector types have been developed so far; the recombinant HSV-1 (rHSV) and the HSV-1 amplicons. rHSV vectors are generated by replacing one or more virus genes with transgene cassettes of interest. HSV-1 amplicon vectors on the other hand contain only two cis-elements from the HSV-1 genome: (i) an origin of DNA replication (e.g. *oriS*) and (ii) a DNA cleavage/packaging signal (*pac*) (Fig. 3.6 A). A plasmid harboring these two genetic elements from HSV-1 is termed amplicon. Such amplicons containing the transgenes of interest are packaged into fully functional HSV-1 virions in cells super-infected with wtHSV-1 (124). One drawback of this method is the massive contamination of vector stocks with wtHSV-1 particles. The generation of a replication and packaging deficient, BAC-cloned HSV-1 genome providing all genes necessary to package amplicons reduced the contamination of amplicon stocks with wtHSV-1 and therefore reduced immunogenicity and toxicity of HSV-1 amplicon stocks (37, 110, 111, 125). Besides the advantages of HSV-1 vectors in general, several HSV-1 amplicon-specific advantages make this vector type a

promising candidate for gene therapy trials: (i) the very large transgene capacity of HSV-based vectors is maintained; (ii) the production of amplicon stocks which are almost 100% free of contaminating wtHSV-1 particles and (iii) the capability to infect most cell types in mammals. However, one major drawback still remains: transgene expression of any HSV-1 based vector type is transient.

To overcome the disadvantages of AAV and HSV-1 based vectors, HSV/AAV hybrid vectors were constructed (48, 60, 142). This vector type is based on the HSV-1 amplicon system vector and therefore conserves all the HSV-1 elements and properties of standard HSV-1 amplicons. But at the same time these hybrid vectors harbor the AAV2 *rep* gene and the AAV2 ITRs flanking the transgene cassette (Fig. 3.6 B). The presence of the AAV2 elements allows the vector to integrate the ITR-flanked transgene cassette into the host genome in a Rep-dependent fashion. This feature circumvents the poor transgene maintenance of transiently expressing HSV-1 amplicon vectors. At the same time HSV/AAV hybrid vectors overcome the small transgene capacity of rAAV vectors. In addition, the presence of the *rep* gene on the same vector but outside of the ITR-cassette assures that *rep* is not integrated and therefore circumvents Rep-mediated toxicity and eventually the rescue of the ITR-cassette from the integrated state. However, some elements of the two hybrid vector partners may interfere with each other and thereby reduce the performance of this vector. In particular, the two origins of DNA replication, e.g. the HSV-1 *oriS*, the AAV2 *ITRs* and the AAV2 *p5* promoter which also functions as an origin of DNA replication (41) may compete for the HSV-1 helper factors and therefore interfere with the HSV-1 mediated replication of the amplicon plasmid, resulting in low packaging efficiency. Moreover, the inevitable expression of the toxic Rep proteins during packaging may have a direct inhibitory effect on HSV-1 replication and therefore contribute to low hybrid-vector titers (41, 42, 48).

The identification of mechanisms how Rep can inhibit HSV-1 replication may contribute to the generation of improved HSV/AAV hybrid vectors.

3.6. Figure Legends & Figures

Figure 3.1. The AAV2 genome organization. Schematic representation of the complete AAV2 genome. On top, the ssDNA is shown in blue. The two ORFs *rep* and *cap* are flanked by two terminal repeats (TR). Two promoters (p5 and p19) are controlling expression of the *rep* ORF, and together with a common splicing site, they are responsible for the expression of the four Rep proteins (Rep78, Rep68, Rep 52 and Rep40). The *cap* gene is transcribed from the p40 promoter and due to alternative start codons leads to the expression of the three capsid proteins (VP1, VP2 and VP3). A non-conventional start codon within the *cap* ORF gives rise to the AAP protein. The transcripts of all proteins are depicted in green and their size is indicated at the right site.

Figure 3.2. Structure of the AAV2 ITR and model of the rolling hairpin mechanism. (A) The structure and the sequence of the AAV2 3'-ITR is represented. The palindromic sequence of the ITR is folded into a hairpin shaped structure. The Rep-binding site, or Rep-binding element (RBE), consists of three perfect GAGC tetranucleotide repeats (open box). The arrow points to the site where Rep cleaves the DNA within the terminal resolution site (trs). The duplexed 3'-end of the genome constitutes the primer for DNA replication, a process called self-priming. **(B)** The diagram represents the model of AAV2 DNA replication. The different steps and structures of the AAV2 genome during DNA replication are indicated. The left half of the model highlights the replication cycle which leads to monomer-length single-stranded progeny genomes. The right half of the model highlights the synthesis of dimer molecules, which are generated when re-initiation occurs before terminal resolution. DNA pol., DNA polymerase; mT, monomer turnaround form; TRS, terminal resolution site; mE, monomer extended form; ss, single-stranded DNA; dT dimer turnaround form; dE, dimer extended form.

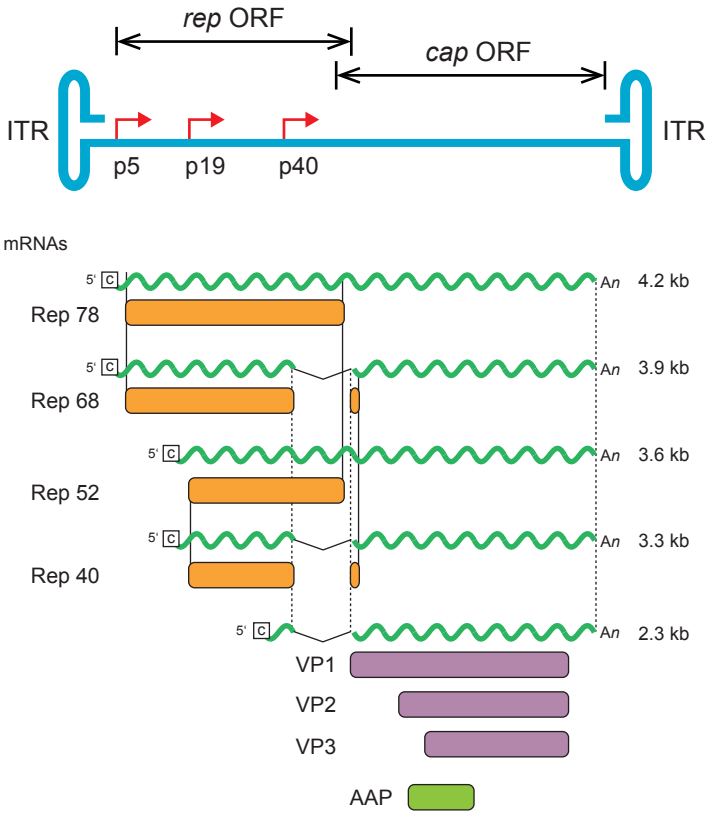
Figure 3.3. Schematic representation of AAV2 Rep functional domains. The complete *rep* ORF and the four different Rep proteins (Rep40, Rep 52, Rep68 and Rep78) are shown on the top of the scheme (grey boxes). The relative location and size of the functional domains are represented below (red boxes) and are discussed in the text. They consist of the DNA-binding domain, the endonuclease domain harbouring the two rolling-circle replication motifs 2 and 3 (RCR2 and RCR3) (yellow boxes), the helicase domain containing the AAA+ domain and the Walker motifs A, B, B' and C (red boxes), as well as the C-terminal PKA binding site containing the PKI like motif (blue boxes).

Figure 3.4. Architecture of the HSV-1 virion particle. The HSV-1 virion is composed of three structural elements: (i) the capsid enclosing the dsDNA genome, (ii) the tegument harbouring a distinct set of viral proteins and (iii) the envelope consisting of a bi-layerd lipid membrane, which is decorated with viral glycoproteins. The diameter of the whole particle varies between 200 and 300 nm. The electronmicrograph is courtesy of E.Schraner, Institute of Virology, University of Zurich. The cryoelectronmicrograph is courtesy of the Max-Planck-Institute of Biochemistry.

Figure 3.5. The HSV-1 genome structure. The HSV-1 genome is a linear double-stranded DNA molecule of approximately 152 kb. It is divided into two distinct segments, unique long (U_L) and unique short (U_S), which are covalently linked via the internal repeats (IR_L and IR_S). The genome contains terminal repeats (TR) at both ends. The origins of DNA replication (*oriL* and *oriS*) as well as the DNA packaging/cleavage signals (*pac*), which are the only *cis*-acting elements required for replication and packaging, are indicated (green and red boxes, respectively).

Figure 3.6. The HSV-1 amplicon and hybrid vector systems. (A) Vero 2-2 cells are co-transfected with the HSV-1 amplicon vector pHSVGFP harboring a transgene cassette encoding for the enhanced green fluorescent protein (EGFP) and replication-competent, but packaging-defective HSV-1 helper DNA (fHSVΔpacΔ27). This helper DNA provides all the functions necessary for replication and packaging of the HSV-amplicon DNA, but due to the lack of the *pac* signal is packaging-defective. The resulting HSV-1 amplicon vector stock is consisting of fully functional HSV-1 virions containing head-to-tail concatemers of the amplicon DNA harboring the transgene cassette. **(B)** The HSV/AAV hybrid vector is based on the HSV-1 amplicon vector as described in panel A. In addition, the HSV/AAV hybrid vector pHyRaNGFPa contains two additional AAV2-specific elements; (i) the AAV2 ITRs which flank the transgene cassette (EGFP) and (ii) the complete *rep* ORF. Vector production follows the same procedure as for the HSV-1 amplicon vector system. The resulting HSV/AAV hybrid vector stocks consist of the same HSV-1 virions as the HSV-1 amplicon stocks, but contain head-to-tail concatemers of the HSV/AAV hybrid vector harboring the additional AAV2 elements to mediate site-specific integration of the transgene cassette.

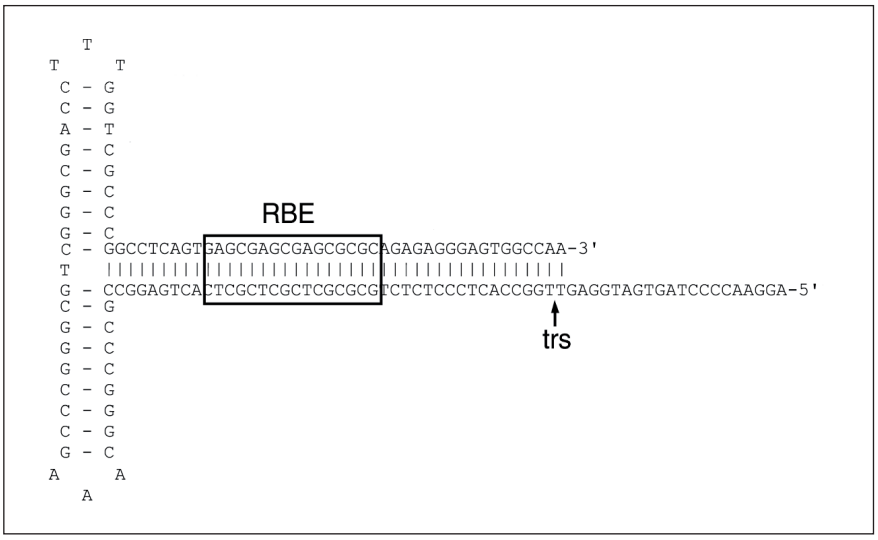
Figure 3.1.



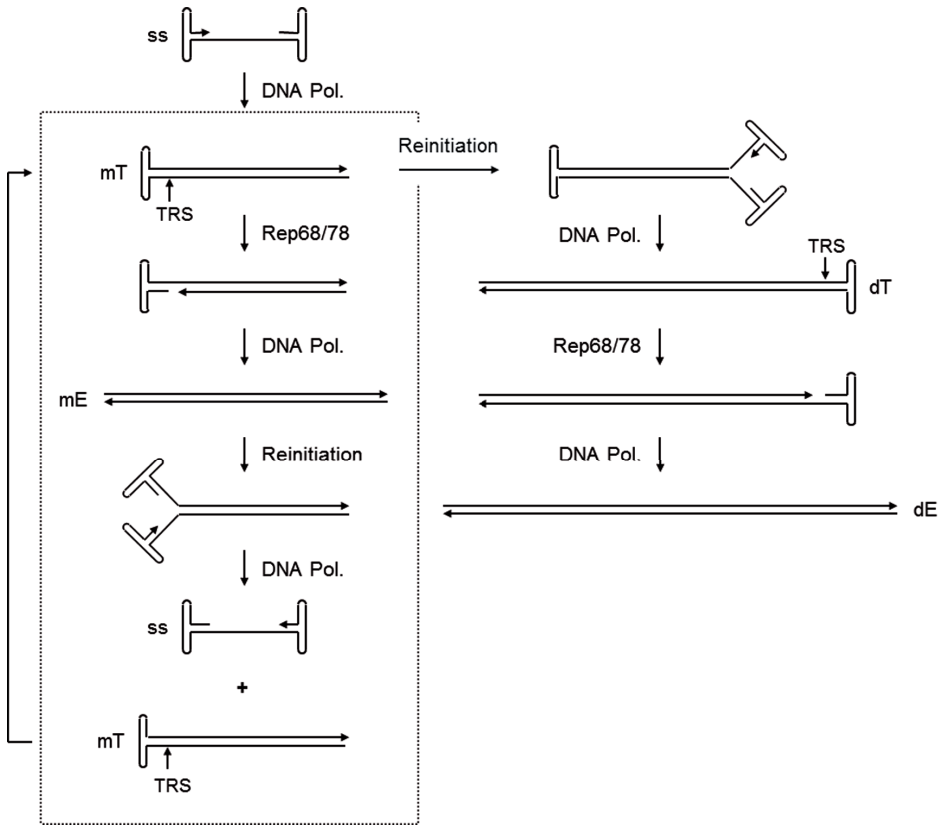
adapted from: Flint et.al, 3rd. edition, 2009

Figure 3.2.

A



B



Recent Advances in DNA Virus Replication, 2006

Figure 3.3.

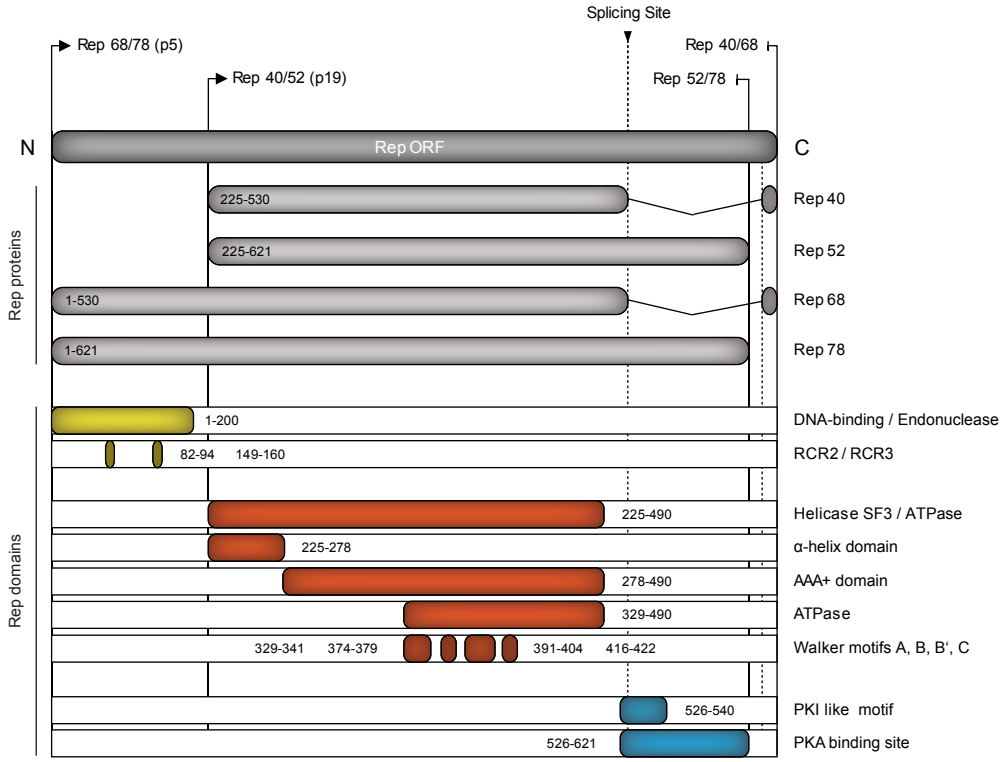
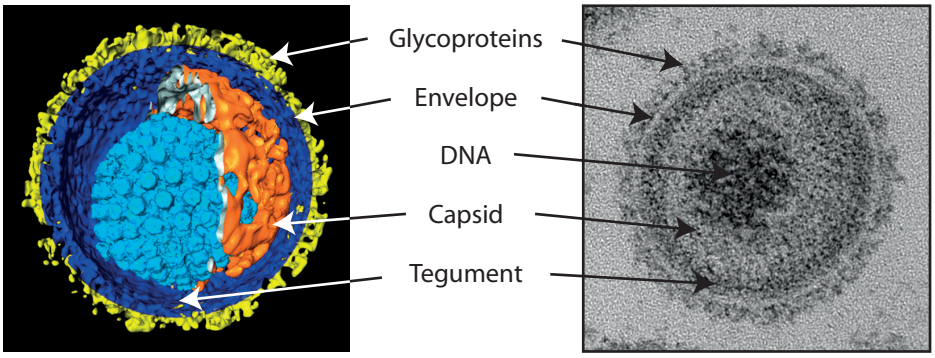

Splicing Site
Rep 68/78 (p5)
Rep 40/52 (p19)
Rep 40/68
Rep 52/78
N
Rep ORF
C
Rep proteins
225-530
Rep 40
225-621
Rep 52
1-530
Rep 68
1-621
Rep 78
Rep domains
1-200
DNA-binding / Endonuclease
82-94
149-160
RCR2 / RCR3
225-490
Helicase SF3 / ATPase
225-278
α-helix domain
278-490
AAA+ domain
329-490
ATPase
329-341
374-379
391-404
416-422
Walker motifs A, B, B', C
526-540
PKI like motif
526-621
PKA binding site

Figure 3.4.



Max-Planck-Institute for Biochemistry, Kay Grünewald

University of Zurich, Institute of Virology, Elisabeth Schraner

Figure 3.5.

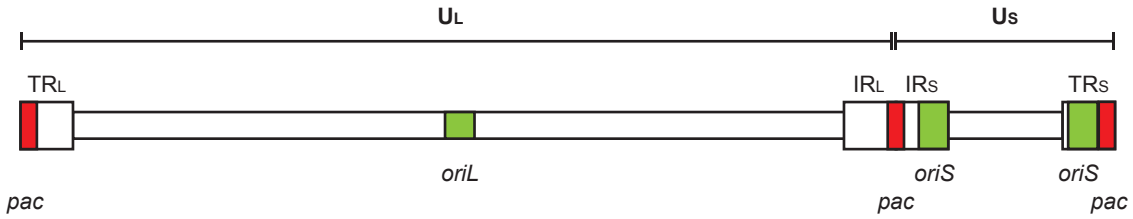
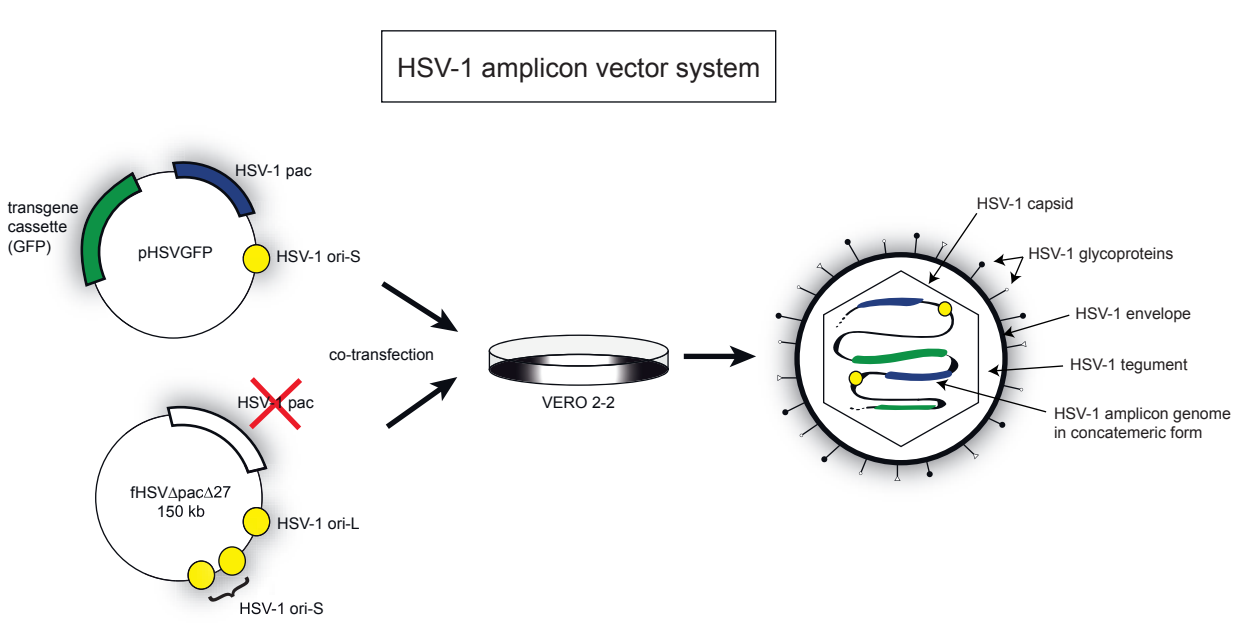
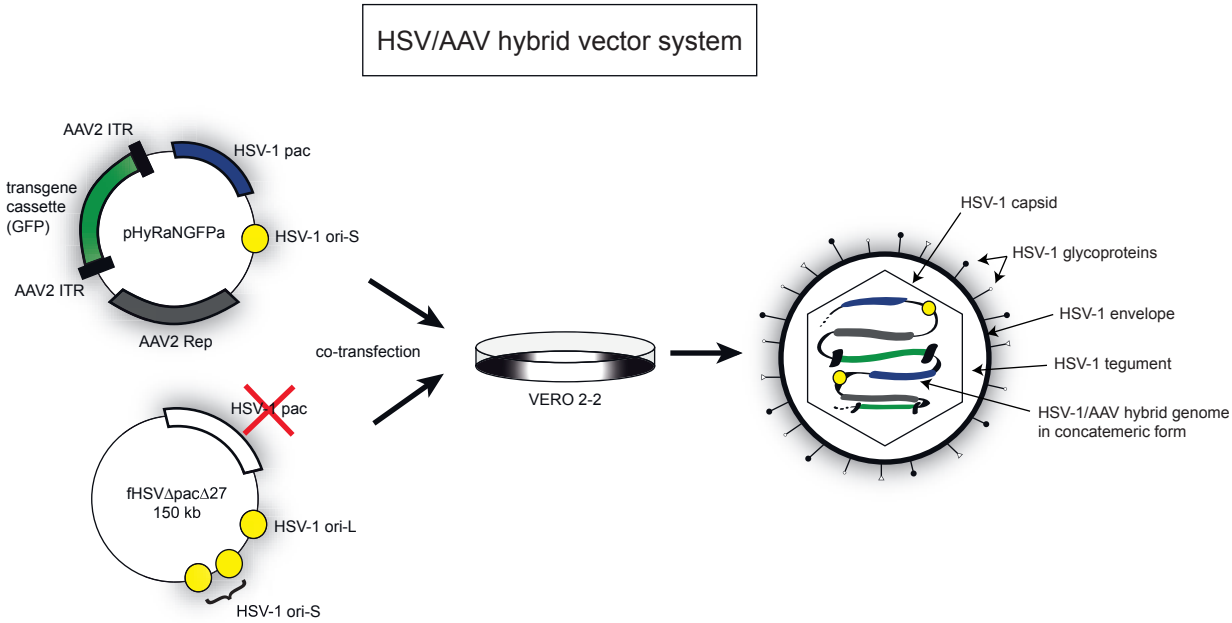


Figure 3.6.

A


HSV-1 amplicon vector system
HSV-1 pac
transgene cassette (GFP)
pHSVGFP
HSV-1 ori-S
co-transfection
HSV-1 pac
fHSVΔpacΔ27 150 kb
HSV-1 ori-L
HSV-1 ori-S
VERO 2-2
HSV-1 capsid
HSV-1 glycoproteins
HSV-1 envelope
HSV-1 tegument
HSV-1 amplicon genome in concatemeric form

B


HSV/AAV hybrid vector system
AAV2 ITR
HSV-1 pac
transgene cassette (GFP)
pHyRaNGFPa
HSV-1 ori-S
AAV2 ITR
AAV2 Rep
co-transfection
HSV-1 pac
fHSVΔpacΔ27 150 kb
HSV-1 ori-L
HSV-1 ori-S
VERO 2-2
HSV-1 capsid
HSV-1 glycoproteins
HSV-1 envelope
HSV-1 tegument
HSV-1/AAV hybrid genome in concatemeric form

3.7. References (Chapter 3)

1. **Alam, S., B. S. Bowser, M. J. Conway, M. Israr, A. Tandon, and C. Meyers.** 2011. Adeno-associated virus type 2 infection activates caspase dependent and independent apoptosis in multiple breast cancer lines but not in normal mammary epithelial cells. Mol Cancer **10**:97.
2. **Atanasiu, D., W. T. Saw, G. H. Cohen, and R. J. Eisenberg.** 2010. Cascade of events governing cell-cell fusion induced by herpes simplex virus glycoproteins gD, gH/gL, and gB. J. Virol. **84**:12292–12299.
3. **Atanasiu, D., J. C. Whitbeck, M. P. de Leon, H. Lou, B. P. Hannah, G. H. Cohen, and R. J. Eisenberg.** 2010. Bimolecular complementation defines functional regions of Herpes simplex virus gB that are involved with gH/gL as a necessary step leading to cell fusion. J. Virol. **84**:3825–3834.
4. **Bantel-Schaal, U., and H. Zur Hausen.** 1988. Adeno-associated viruses inhibit SV40 DNA amplification and replication of herpes simplex virus in SV40-transformed hamster cells. Virology **164**:64–74.
5. **Bartlett, J. S., R. Wilcher, and R. J. Samulski.** 2000. Infectious entry pathway of adeno-associated virus and adeno-associated virus vectors. J Virol **74**:2777–2785. "http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=10684294 ".
6. **Becerra, S. P., F. Koczot, P. Fabisch, and J. A. Rose.** 1988. Synthesis of adeno-associated virus structural proteins requires both alternative mRNA splicing and alternative initiations from a single transcript. J Virol **62**:2745–2754.
7. **Becerra, S. P., J. A. Rose, M. Hardy, B. M. Baroudy, and C. W. Anderson.** 1985. Direct mapping of adeno-associated virus capsid proteins B and C: a possible ACG initiation codon. Proc Natl Acad Sci U S A **82**:7919–7923.
8. **Bernard Roizman, and Philip E. Pellett.** 2001. The Family herpesviridae: A Brief Introduction, p. 2381–2397. *In* D. M. Knipe and P. M. Howley (ed.), Fields Virology. Lippincott Williams & Wilkins, 4th ed., vol. 2. Lippincott Williams & Wilkins, Philadelphia.
9. **Berns, K. I., and C. Giraud.** 1996. Biology of adeno-associated virus. Curr. Top. Microbiol. Immunol. **218**:1–23.
10. **Berns, K. I., and W. W. Hauswirth.** 1979. Adeno-associated viruses. Adv. Virus Res. **25**:407–449.
11. **Berns, K. I., W. W. Hauswirth, K. H. Fife, and E. Lusby.** 1979. Adeno-associated virus DNA replication. Cold Spring Harb. Symp. Quant. Biol. **43 Pt 2**:781–787.

12. **Blacklow, N. R., R. Dolin, and M. D. Hoggan.** 1971. Studies of the enhancement of an adenovirus-associated virus by herpes simplex virus. Journal of General Virology **10**:29–36.
13. **Brister, J. R., and N. Muzyczka.** 2000. Mechanism of Rep-mediated adeno-associated virus origin nicking. Journal of Virology **74**:7762–7771.
14. **Browne, H. M.** 2009. The role of glycoprotein H in herpesvirus membrane fusion. Protein Pept. Lett. **16**:760–765.
15. **Bryant, L. M., D. M. Christopher, A. R. Giles, C. Hinderer, J. L. Rodriguez, J. B. Smith, E. A. Traxler, J. Tycko, A. P. Wojno, and J. M. Wilson.** 2013. Lessons learned from the clinical development and market authorization of Glybera. Hum Gene Ther Clin Dev **24**:55–64.
16. **Buller, R. M., J. E. Janik, E. D. Sebring, and J. A. Rose.** 1981. Herpes simplex virus types 1 and 2 completely help adenovirus-associated virus replication. Journal of Virology **40**:241–247.
17. **Burkham, J., D. M. Coen, C. B. Hwang, and S. K. Weller.** 2001. Interactions of herpes simplex virus type 1 with ND10 and recruitment of PML to replication compartments. Journal of Virology **75**:2353–2367.
18. **Burnett, J. R., and A. J. Hooper.** 2009. Alipogene tiparvovec, an adeno-associated virus encoding the Ser(447)X variant of the human lipoprotein lipase gene for the treatment of patients with lipoprotein lipase deficiency. Curr. Opin. Mol. Ther. **11**:681–691.
19. **Casper, J. M., J. M. Timpe, J. D. Dignam, and J. P. Trempe.** 2005. Identification of an adeno-associated virus Rep protein binding site in the adenovirus E2a promoter. Journal of Virology **79**:28–38.
20. **Cassinotti, P., M. Weitzand, and J. D. Tratschin.** 1988. Organization of the adeno-associated virus (AAV) capsid gene: mapping of a minor spliced mRNA coding for virus capsid protein. Virology **167**:176–184.
21. **Chang, L. S., Y. Shi, and T. Shenk.** 1989. Adeno-associated virus P5 promoter contains an adenovirus E1A-inducible element and a binding site for the major late transcription factor. Journal of Virology **63**:3479–3488.
22. **Chayavichitsilp, P., J. V. Buckwalter, A. C. Krakowski, and S. F. Friedlander.** 2009. Herpes simplex. Pediatr Rev **30**:119-29; quiz 130.
23. **Chejanovsky, N., and B. J. Carter.** 1989. Mutagenesis of an AUG codon in the adeno-associated virus rep gene: effects on viral DNA replication. Virology **173**:120–128.

24. **Chen, S.-H., Y.-W. Lin, A. Griffiths, W.-Y. Huang, and S.-H. Chen.** 2006. Competition and complementation between thymidine kinase-negative and wild-type herpes simplex virus during co-infection of mouse trigeminal ganglia. J Gen Virol **87**:3495–3502.
25. **Cheung, A. K., M. D. Hoggan, W. W. Hauswirth, and K. I. Berns.** 1980. Integration of the adeno-associated virus genome into cellular DNA in latently infected human Detroit 6 cells. J. Virol. **33**:739–748.
26. **Chiorini, J. A., S. M. Wiener, R. A. Owens, S. R. Kyostio, R. M. Kotin, and B. Safer.** 1994. Sequence requirements for stable binding and function of Rep68 on the adeno-associated virus type 2 inverted terminal repeats. Journal of Virology **68**:7448–7457.
27. **Davis, M. D., J. Wu, and R. A. Owens.** 2000. Mutational analysis of adeno-associated virus type 2 Rep68 protein endonuclease activity on partially single-stranded substrates. J Virol **74**:2936–2942. "http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db= PubMed&dopt=Citation&list_uids=10684315 ".
28. **Deiss, L. P., J. Chou, and N. Frenkel.** 1986. Functional domains within the a sequence involved in the cleavage-packaging of herpes simplex virus DNA. Journal of Virology **59**:605–618.
29. **Di Pasquale, G., and J. A. Chiorini.** 2003. PKA/PrKX activity is a modulator of AAV/adenovirus interaction. EMBO Journal **22**:1716–1724.
30. **Dohner, K., A. Wolfstein, U. Prank, C. Echeverri, D. Dujardin, R. Vallee, and B. Sodeik.** 2002. Function of dynein and dynactin in herpes simplex virus capsid transport. Molecular Biology of the Cell **13**:2795–2809.
31. **Duan, D., Q. Li, A. W. Kao, Y. Yue, J. E. Pessin, and J. F. Engelhardt.** 1999. Dynamin is required for recombinant adeno-associated virus type 2 infection. Journal of Virology **73**:10371–10376.
32. **Duan, D., P. Sharma, J. Yang, Y. Yue, L. Dudus, Y. Zhang, K. J. Fisher, and J. F. Engelhardt.** 1998. Circular intermediates of recombinant adeno-associated virus have defined structural characteristics responsible for long-term episomal persistence in muscle tissue. J. Virol. **72**:8568–8577.
33. **Dutheil, N., F. Shi, T. Dupressoir, and R. M. Linden.** 2000. Adeno-associated virus site-specifically integrates into a muscle-specific DNA region. Proceedings of the National Academy of Sciences of the United States of America **97**:4862–4866.
34. **Everett, R. D., C. Boutell, and A. Orr.** 2004. Phenotype of a herpes simplex virus type 1 mutant that fails to express immediate-early regulatory protein ICP0. J Virol **78**:1763–1774.

"http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=14747541 ".

35. **Fields, B. N., D. M. Knipe, and P. M. Howley** (ed.). 2007. Fields' virology, 5th ed. /. Wolters Kluwer/Lippincott Williams & Wilkins, Philadelphia, Pa., London. http://www.loc.gov/catdir/toc/ecip072/2006032230.html/ http://www.loc.gov/catdir/enhancements/fy0712/2006032230-d.html.
36. **Fisher, K. J., G. P. Gao, M. D. Weitzman, R. DeMatteo, J. F. Burda, and J. M. Wilson.** 1996. Transduction with recombinant adeno-associated virus for gene therapy is limited by leading-strand synthesis. Journal of Virology **70**:520–532.
37. **Fraefel, C., S. Song, F. Lim, P. Lang, L. Yu, Y. Wang, P. Wild, and A. I. Geller.** 1996. Helper virus-free transfer of herpes simplex virus type 1 plasmid vectors into neural cells. Journal of Virology **70**:7190–7197.
38. **Geoffroy, M. C., G. Chadeuf, A. Orr, A. Salvetti, and R. D. Everett.** 2006. Impact of the interaction between herpes simplex virus type 1 regulatory protein ICP0 and ubiquitin-specific protease USP7 on activation of adeno-associated virus type 2 rep gene expression. Journal of Virology **80**:3650–3654.
39. **Geoffroy, M. C., A. L. Epstein, E. Toublanc, P. Moullier, and A. Salvetti.** 2004. Herpes simplex virus type 1 ICP0 protein mediates activation of adeno-associated virus type 2 rep gene expression from a latent integrated form. Journal of Virology **78**:10977–10986.
40. **Giraud, C., E. Winocour, and K. I. Berns.** 1994. Site-specific integration by adeno-associated virus is directed by a cellular DNA sequence. Proceedings of the National Academy of Sciences of the United States of America **91**:10039–10043.
41. **Glauser, D. L., O. Saydam, N. A. Balsiger, I. Heid, R. M. Linden, M. Ackermann, and C. Fraefel.** 2005. Four-dimensional visualization of the simultaneous activity of alternative adeno-associated virus replication origins. Journal of Virology **79**:12218–12230.
42. **Glauser, D. L., M. Seyffert, R. Strasser, M. Franchini, A. S. Laimbacher, C. Dresch, A. P. de Oliveira, R. Vogel, H. Büning, A. Salvetti, M. Ackermann, and C. Fraefel.** 2010. Inhibition of herpes simplex virus type 1 replication by adeno-associated virus rep proteins depends on their combined DNA-binding and ATPase/helicase activities. J. Virol. **84**:3808–3824. doi:10.1128/JVI.01503-09.
43. **Glauser, D. L., R. Strasser, A. S. Laimbacher, O. Saydam, N. Clément, R. M. Linden, M. Ackermann, and C. Fraefel.** 2007. Live covisualization of competing

adeno-associated virus and herpes simplex virus type 1 DNA replication: molecular mechanisms of interaction. Journal of Virology **81**:4732–4743.

44. **Goncalves, M. A. F. V.** 2005. Adeno-associated virus: from defective virus to effective vector. Virology Journal **2**.
45. **Gorbalenya, A. E., E. V. Koonin, and Y. I. Wolf.** 1990. A new superfamily of putative NTP-binding domains encoded by genomes of small DNA and RNA viruses. FEBS Letters **262**:145–148.
46. **Hansen, J., K. Qing, H. J. Kwon, C. Mah, and A. Srivastava.** 2000. Impaired intracellular trafficking of adeno-associated virus type 2 vectors limits efficient transduction of murine fibroblasts. Journal of Virology **74**:992–996.
47. **Heilbronn, R., A. Burkle, S. Stephan, and H. Zur Hausen.** 1990. The adeno-associated virus rep gene suppresses herpes simplex virus-induced DNA amplification. Journal of Virology **64**:3012–3018.
48. **Heister, T., I. Heid, M. Ackermann, and C. Fraefel.** 2002. Herpes simplex virus type 1/adeno-associated virus hybrid vectors mediate site-specific integration at the adeno-associated virus preintegration site, AAVS1, on human chromosome 19. Journal of Virology **76**:7163–7173.
49. **Henckaerts, E., N. Dutheil, N. Zeltner, S. Kattman, E. Kohlbrenner, P. Ward, N. Clément, P. Rebollo, M. Kennedy, G. M. Keller, and R. M. Linden.** 2009. Site-specific integration of adeno-associated virus involves partial duplication of the target locus. Proc. Natl. Acad. Sci. U.S.A. **106**:7571–7576. doi:10.1073/pnas.0806821106.
50. **Hickman, A. B., and F. Dyda.** 2005. Binding and unwinding: SF3 viral helicases. Curr. Opin. Struct. Biol. **15**:77–85. doi:10.1016/j.sbi.2004.12.001.
51. **Hickman, A. B., D. R. Ronning, R. M. Kotin, and F. Dyda.** 2002. Structural unity among viral origin binding proteins: crystal structure of the nuclease domain of adeno-associated virus Rep. J. Virol. **10**:327–337.
52. **Hickman, A. B., D. R. Ronning, Z. N. Perez, R. M. Kotin, and F. Dyda.** 2004. The nuclease domain of adeno-associated virus rep coordinates replication initiation using two distinct DNA recognition interfaces. Molecular Cell **13**:403–414.
53. **Hoggan, M. D., N. R. Blacklow, and W. P. Rowe.** 1966. Studies of small DNA viruses found in various adenovirus preparations: physical, biological, and immunological characteristics. Proceedings of the National Academy of Sciences of the United States of America **55**:1467–1474.
54. **Hong, G., P. Ward, and K. I. Berns.** 1994. Intermediates of adeno-associated virus DNA replication in vitro. Journal of Virology **68**:2011–2015.

55. **Horer, M., S. Weger, K. Butz, F. Hoppe-Seyler, C. Geisen, and J. A. Kleinschmidt.** 1995. Mutational analysis of adeno-associated virus Rep protein-mediated inhibition of heterologous and homologous promoters. J Virol **69**:5485–5496.
56. **Im, D. S., and N. Muzyczka.** 1989. Factors that bind to adeno-associated virus terminal repeats. Journal of Virology **63**:3095–3104.
57. **Iyer, L. M., K. S. Makarova, E. V. Koonin, and L. Aravind.** 2004. Comparative genomics of the FtsK-HerA superfamily of pumping ATPases: implications for the origins of chromosome segregation, cell division and viral capsid packaging. Nucleic Acids Res. **32**:5260–5279.
58. **Janik, J. E., M. M. Huston, and J. A. Rose.** 1981. Locations of adenovirus genes required for the replication of adenovirus-associated virus. Proceedings of the National Academy of Sciences of the United States of America **78**:1925–1929.
59. **Jay, F. T., C. A. Laughlin, and B. J. Carter.** 1981. Eukaryotic translational control: adeno-associated virus protein synthesis is affected by a mutation in the adenovirus DNA-binding protein. Proc. Natl. Acad. Sci. U.S.A. **78**:2927–2931.
60. **Johnston, K. M., D. Jacoby, P. A. Pechan, C. Fraefel, P. Borghesani, D. Schuback, R. J. Dunn, F. I. Smith, and X. O. Breakefield.** 1997. HSV/AAV hybrid amplicon vectors extend transgene expression in human glioma cells. Human Gene Therapy **8**:359–370.
61. **Kashiwakura, Y., K. Tamayose, K. Iwabuchi, Y. Hirai, T. Shimada, K. Matsumoto, T. Nakamura, M. Watanabe, K. Oshimi, and H. Daida.** 2005. Hepatocyte growth factor receptor is a coreceptor for adeno-associated virus type 2 infection. Journal of Virology **79**:609–614.
62. **Kelly, B. J., C. Fraefel, A. L. Cunningham, and R. J. Diefenbach.** 2009. Functional roles of the tegument proteins of herpes simplex virus type 1. Virus Research **145**:173–186.
63. **King, J. A.** 2001. DNA helicase-mediated packaging of adeno-associated virus type 2 genomes into preformed capsids. The EMBO Journal **20**:3282–3291.
64. **Kleinschmidt, J. A., M. Mohler, F. W. Weindler, and R. Heilbronn.** 1995. Sequence elements of the adeno-associated virus rep gene required for suppression of herpes-simplex-virus-induced DNA amplification. Virology **206**:254–262.
65. **Kotin, R. M., and K. I. Berns.** 1989. Organization of adeno-associated virus DNA in latently infected Detroit 6 cells. Virology **170**:460–467.

66. **Kotin, R. M., R. M. Linden, and K. I. Berns.** 1992. Characterization of a preferred site on human chromosome 19q for integration of adeno-associated virus DNA by non-homologous recombination. EMBO Journal **11**:5071–5078.
67. **Kotin, R. M., J. C. Menninger, D. C. Ward, and K. I. Berns.** 1991. Mapping and direct visualization of a region-specific viral DNA integration site on chromosome 19q13-qter. Genomics **10**:831–834.
68. **Kotin, R. M., M. Siniscalco, R. J. Samulski, X. D. Zhu, L. Hunter, C. A. Laughlin, S. McLaughlin, N. Muzyczka, M. Rocchi, and K. I. Berns.** 1990. Site-specific integration by adeno-associated virus. Proceedings of the National Academy of Sciences of the United States of America **87**:2211–2215.
69. **Kyostio, S. R., R. A. Owens, M. D. Weitzman, B. A. Antoni, N. Chejanovsky, and B. J. Carter.** 1994. Analysis of adeno-associated virus (AAV) wild-type and mutant Rep proteins for their abilities to negatively regulate AAV p5 and p19 mRNA levels. Journal of Virology **68**:2947–2957.
70. **Kyostio, S. R., R. S. Wonderling, and R. A. Owens.** 1995. Negative regulation of the adeno-associated virus (AAV) P5 promoter involves both the P5 rep binding site and the consensus ATP-binding motif of the AAV Rep68 protein. Journal of Virology **69**:6787–6796.
71. **Labow, M. A., P. L. Hermonat, and K. I. Berns.** 1986. Positive and negative autoregulation of the adeno-associated virus type 2 genome. Journal of Virology **60**:251–258.
72. **Laughlin, C. A., N. Jones, and B. J. Carter.** 1982. Effect of deletions in adenovirus early region 1 genes upon replication of adeno-associated virus. Journal of Virology **41**:868–876.
73. **Laughlin, C. A., J. D. Tratschin, H. Coon, and B. J. Carter.** 1983. Cloning of infectious adeno-associated virus genomes in bacterial plasmids. Gene **23**:65–73.
74. **Lilley, C. E., C. T. Carson, A. R. Muotri, F. H. Gage, and M. D. Weitzman.** 2005. DNA repair proteins affect the lifecycle of herpes simplex virus 1. Proceedings of the National Academy of Sciences of the United States of America **102**:5844–5849.
75. **Linden, R. M., and K. I. Berns.** 2000. Molecular biology of adeno-associated viruses, vol. 4.
76. **Linden, R. M., P. Ward, C. Giraud, E. Winocour, and K. I. Berns.** 1996. Site-specific integration by adeno-associated virus. Proceedings of the National Academy of Sciences of the United States of America **93**:11288–11294.

77. **Linden, R. M., E. Winocour, and K. I. Berns.** 1996. The recombination signals for adeno-associated virus site-specific integration. Proceedings of the National Academy of Sciences of the United States of America **93**:7966–7972.
78. **Liptak, L. M., S. L. Uprichard, and D. M. Knipe.** 1996. Functional order of assembly of herpes simplex virus DNA replication proteins into prereplicative site structures. J. Virol. **70**:1759–1767.
79. **Liu, X., K. Fitzgerald, E. Kurt-Jones, R. Finberg, and D. M. Knipe.** 2008. Herpesvirus tegument protein activates NF-kappaB signaling through the TRAF6 adaptor protein. Proc Natl Acad Sci U S A **105**:11335–11339.
80. **Li, Z., J. R. Brister, D. S. Im, and N. Muzyczka.** 2003. Characterization of the adenoassociated virus Rep protein complex formed on the viral origin of DNA replication. Virology **313**:364–376.
81. **Lukonis, C. J., and S. K. Weller.** 1996. Characterization of nuclear structures in cells infected with herpes simplex virus type 1 in the absence of viral DNA replication. Journal of Virology **70**:1751–1758.
82. **Lukonis, C. J., and S. K. Weller.** 1997. Formation of herpes simplex virus type 1 replication compartments by transfection: requirements and localization to nuclear domain 10. Journal of Virology **71**:2390–2399.
83. **Luxton, G. W., S. Haverlock, K. E. Coller, S. E. Antinone, A. Pincetic, and G. A. Smith.** 2005. Targeting of herpesvirus capsid transport in axons is coupled to association with specific sets of tegument proteins. Proc Natl Acad Sci U S A **102**:5832–5837. "http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=15795370 ".
84. **McCarty, D. M., D. J. Pereira, I. Zolotukhin, X. Zhou, J. H. Ryan, and N. Muzyczka.** 1994. Identification of linear DNA sequences that specifically bind the adeno-associated virus Rep protein. Journal of Virology **68**:4988–4997.
85. **McCarty, D. M., J. H. Ryan, S. Zolotukhin, X. Zhou, and N. Muzyczka.** 1994. Interaction of the adeno-associated virus Rep protein with a sequence within the A palindrome of the viral terminal repeat. Journal of Virology **68**:4998–5006.
86. **McCarty, D. M., S. M. Young, JR., and R. J. Samulski.** 2004. Integration of adeno-associated virus (AAV) and recombinant AAV vectors. Annual Review of Genetics **38**:819–845.
87. **McPherson, R. A., L. J. Rosenthal, and J. A. Rose.** 1985. Human cytomegalovirus completely helps adeno-associated virus replication. Virology **147**:217–222.

88. **Mishra, L., and J. A. Rose.** 1990. Adeno-associated virus DNA replication is induced by genes that are essential for HSV-1 DNA synthesis. Virology **179**:632–639.
89. **Mohni, K. N., A. R. Dee, S. Smith, A. J. Schumacher, and S. K. Weller.** 2012. Efficient Herpes Simplex Virus 1 Replication Requires Cellular ATR Pathway Proteins. Journal of Virology **87**:531–542.
90. **Mohni, K. N., C. M. Livingston, D. Cortez, and S. K. Weller.** 2010. ATR and ATRIP Are Recruited to Herpes Simplex Virus Type 1 Replication Compartments Even though ATR Signaling Is Disabled. Journal of Virology **84**:12152–12164.
91. **Muralidhar, S., S. P. Becerra, and J. A. Rose.** 1994. Site-directed mutagenesis of adeno-associated virus type 2 structural protein initiation codons: effects on regulation of synthesis and biological activity. Journal of Virology **68**:170–176.
92. **Muzyczka, N.** 1992. Use of adeno-associated virus as a general transduction vector for mammalian cells. Current Topics in Microbiology & Immunology **158**:97–129.
93. **Muzyczka, N., and K. I. Berns.** 2001. Parvoviridae: The viruses and their replication, p. 2327–2346. *In* D. M. Knipe and P. M. Howley (ed.), Fields Virology. Lippincott Williams & Wilkins, 4th ed., vol. 2. Lippincott Williams & Wilkins, Philadelphia.
94. **Nash, Chen, Salganik, and Muzyczka.** 2008. Identification of Cellular Proteins that interact with the Adeno-Associated Virus Rep protein. J. Virol. doi:10.1128/JVI.01939-08.
95. **Naumer, M., F. Sonntag, K. Schmidt, K. Nieto, C. Panke, N. E. Davey, R. Popa-Wagner, and J. A. Kleinschmidt.** 2012. Properties of the Adeno-Associated Virus Assembly-Activating Protein. Journal of Virology **86**:13038–13048.
96. **Needham, P. G., J. M. Casper, V. Kalman-Maltese, K. Verrill, J. D. Dignam, and J. P. Trempe.** 2006. Adeno-associated virus rep protein-mediated inhibition of transcription of the adenovirus major late promoter in vitro. Journal of Virology **80**:6207–6217.
97. **Nicolas, A., N. Alazard-Dany, C. Biollay, L. Arata, N. Jolinon, L. Kuhn, M. Ferro, S. K. Weller, A. L. Epstein, A. Salvetti, and A. Greco.** 2010. Identification of Rep-Associated Factors in Herpes Simplex Virus Type 1-Induced Adeno-Associated Virus Type 2 Replication Compartments. Journal of Virology **84**:8871–8887.
98. **Ni, T. H., W. F. McDonald, I. Zolotukhin, T. Melendy, S. Waga, B. Stillman, and N. Muzyczka.** 1998. Cellular proteins required for adeno-associated virus DNA replication in the absence of adenovirus coinfection. Journal of Virology **72**:2777–2787.
99. **Ni, T. H., X. Zhou, D. M. McCarty, I. Zolotukhin, and N. Muzyczka.** 1994. In vitro replication of adeno-associated virus DNA. Journal of Virology **68**:1128–1138.

100. **Owens, R. A., M. D. Weitzman, S. R. Kyostio, and B. J. Carter.** 1993. Identification of a DNA-binding domain in the amino terminus of adeno-associated virus Rep proteins. Journal of Virology **67**:997–1005.
101. **Pereira, D. J., D. M. McCarty, and N. Muzyczka.** 1997. The adeno-associated virus (AAV) Rep protein acts as both a repressor and an activator to regulate AAV transcription during a productive infection. Journal of Virology **71**:1079–1088.
102. **Perez, A., Q. X. Li, P. Perez-Romero, G. Delassus, S. R. Lopez, S. Sutter, N. McLaren, and A. O. Fuller.** 2005. A new class of receptor for herpes simplex virus has heptad repeat motifs that are common to membrane fusion proteins. Journal of Virology **79**:7419–7430.
103. **Pertel, P. E., A. Fridberg, M. L. Parish, and P. G. Spear.** 2001. Cell fusion induced by herpes simplex virus glycoproteins gB, gD, and gH-gL requires a gD receptor but not necessarily heparan sulfate. Virology **279**:313–324.
104. **Qing, K., C. Mah, J. Hansen, S. Zhou, V. Dwarki, and A. Srivastava.** 1999. Human fibroblast growth factor receptor 1 is a co-receptor for infection by adeno-associated virus 2.[see comment]. Nature Medicine **5**:71–77.
105. **Reske, A., G. Pollara, C. Krummenacher, B. M. Chain, and D. R. Katz.** 2007. Understanding HSV-1 entry glycoproteins. Rev. Med. Virol. **17**:205–215.
106. **Richardson, W. D., and H. Westphal.** 1984. Requirement for either early region 1a or early region 1b adenovirus gene products in the helper effect for adeno-associated virus. Journal of Virology **51**:404–410.
107. **Rock, D. L., A. B. Nesburn, H. Ghiasi, J. Ong, T. L. Lewis, J. R. Lokensgard, and S. L. Wechsler.** 1987. Detection of latency-related viral RNAs in trigeminal ganglia of rabbits latently infected with herpes simplex virus type 1. Journal of Virology **61**:3820–3826.
108. **Roizman, B., and D. M. Knipe.** 2001. Herpes simplex viruses and their replication, p. 2399–2459. *In* D. M. Knipe and P. M. Howley (ed.), Fields Virology. Lippincott Williams & Wilkins, 4th ed., vol. 2. Lippincott Williams & Wilkins, Philadelphia.
109. **Ryan, J. H., S. Zolotukhin, and N. Muzyczka.** 1996. Sequence requirements for binding of Rep68 to the adeno-associated virus terminal repeats. Journal of Virology **70**:1542–1553.
110. **Saeki, Y., C. Fraefel, T. Ichikawa, X. O. Breakefield, and E. A. Chiocca.** 2001. Improved helper virus-free packaging system for HSV amplicon vectors using an ICP27-deleted, oversized HSV-1 DNA in a bacterial artificial chromosome. Molecular Therapy: the Journal of the American Society of Gene Therapy **3**:591–601.

111. **Saeki, Y., T. Ichikawa, A. Saeki, E. A. Chiocca, K. Tobler, M. Ackermann, X. O. Breakefield, and C. Fraefel.** 1998. Herpes simplex virus type 1 DNA amplified as bacterial artificial chromosome in Escherichia coli: rescue of replication-competent virus progeny and packaging of amplicon vectors. Human Gene Therapy **9**:2787–2794.
112. **Samulski, R. J., K. I. Berns, M. Tan, and N. Muzyczka.** 1982. Cloning of adeno-associated virus into pBR322: rescue of intact virus from the recombinant plasmid in human cells. Proceedings of the National Academy of Sciences of the United States of America **79**:2077–2081.
113. **Samulski, R. J., A. Srivastava, K. I. Berns, and N. Muzyczka.** 1983. Rescue of adeno-associated virus from recombinant plasmids: gene correction within the terminal repeats of AAV. Cell **33**:135–143.
114. **Samulski, R. J., X. Zhu, X. Xiao, J. D. Brook, D. E. Housman, N. Epstein, and L. A. Hunter.** 1991. Targeted integration of adeno-associated virus (AAV) into human chromosome 19.[erratum appears in EMBO J 1992 Mar;11(3):1228]. EMBO Journal **10**:3941–3950.
115. **Sanlioglu, S., P. K. Benson, J. Yang, E. M. Atkinson, T. Reynolds, and J. F. Engelhardt.** 2000. Endocytosis and nuclear trafficking of adeno-associated virus type 2 are controlled by rac1 and phosphatidylinositol-3 kinase activation. Journal of Virology **74**:9184–9196.
116. **Saudan, P., J. Vlach, and P. Beard.** 2000. Inhibition of S-phase progression by adeno-associated virus Rep78 protein is mediated by hypophosphorylated pRb. EMBO Journal **19**:4351–4361.
117. **Shirata, N., A. Kudoh, T. Daikoku, Y. Tatsumi, M. Fujita, T. Kiyono, Y. Sugaya, H. Isomura, K. Ishizaki, and T. Tsurumi.** 2005. Activation of ataxia telangiectasia-mutated DNA damage checkpoint signal transduction elicited by herpes simplex virus infection. Journal of Biological Chemistry **280**:30336–30341.
118. **Shukla, D., and P. G. Spear.** 2001. Herpesviruses and heparan sulfate: an intimate relationship in aid of viral entry. J. Clin. Invest. **108**:503–510.
119. **Singleton, M. R., M. S. Dillingham, and D. B. Wigley.** 2007. Structure and mechanism of helicases and nucleic acid translocases. Annu Rev Biochem **76**:23–50.
120. **Smith, R. H., and R. M. Kotin.** 2000. An adeno-associated virus (AAV) initiator protein, Rep78, catalyzes the cleavage and ligation of single-stranded AAV ori DNA. J Virol **74**:3122–3129.
"http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=10708427 ".

121. **Sodeik, B., M. W. Ebersold, and A. Helenius.** 1997. Microtubule-mediated transport of incoming herpes simplex virus 1 capsids to the nucleus. Journal of Cell Biology **136**:1007–1021.
122. **Sonntag, F., K. Schmidt, and J. A. Kleinschmidt.** 2010. A viral assembly factor promotes AAV2 capsid formation in the nucleolus. Proc. Natl. Acad. Sci. U.S.A. **107**:10220–10225. doi:10.1073/pnas.1001673107.
123. **Sourvinos, G., and R. D. Everett.** 2002. Visualization of parental HSV-1 genomes and replication compartments in association with ND10 in live infected cells. EMBO Journal **21**:4989–4997.
124. **Spaete, R. R., and N. Frenkel.** 1982. The herpes simplex virus amplicon: a new eucaryotic defective-virus cloning-amplifying vector. Cell **30**:295–304.
125. **Stavropoulos, T. A., and C. A. Strathdee.** 1998. An enhanced packaging system for helper-dependent herpes simplex virus vectors. Journal of Virology **72**:7137–7143.
126. **Stevens, J. G.** 1975. Latent herpes simplex virus and the nervous system. Current Topics in Microbiology & Immunology **70**:31–50.
127. **Stow, N. D.** 1982. Localization of an origin of DNA replication within the TRS/IRS repeated region of the herpes simplex virus type 1 genome. EMBO Journal **1**:863–867.
128. **Stracker, T. H., G. D. Cassell, P. Ward, Y. M. Loo, B. van Breukelen, S. D. Carrington-Lawrence, R. K. Hamatake, P. C. van der Vliet, S. K. Weller, T. Melendy, and M. D. Weitzman.** 2004. The Rep protein of adeno-associated virus type 2 interacts with single-stranded DNA-binding proteins that enhance viral replication. Journal of Virology **78**:441–453.
129. **Summerford, C., J. S. Bartlett, and R. J. Samulski.** 1999. AlphaVbeta5 integrin: a co-receptor for adeno-associated virus type 2 infection.[see comment]. Nature Medicine **5**:78–82.
130. **Summerford, C., and R. J. Samulski.** 1998. Membrane-associated heparan sulfate proteoglycan is a receptor for adeno-associated virus type 2 virions. Journal of Virology **72**:1438–1445.
131. **Surosky, R. T., M. Urabe, S. G. Godwin, S. A. McQuiston, G. J. Kurtzman, K. Ozawa, and G. Natsoulis.** 1997. Adeno-associated virus Rep proteins target DNA sequences to a unique locus in the human genome. Journal of Virology **71**:7951–7959.
132. **Tattersall, P., and D. C. Ward.** 1976. Rolling hairpin model for replication of parvovirus and linear chromosomal DNA. Nature **263**:106–109.
133. **Thomson, B. J., F. W. Weindler, D. Gray, V. Schwaab, and R. Heilbronn.** 1994. Human herpesvirus 6 (HHV-6) is a helper virus for adeno-associated virus type 2 (AAV-

2) and the AAV-2 rep gene homologue in HHV-6 can mediate AAV-2 DNA replication and regulate gene expression. Virology **204**:304–311. doi:10.1006/viro.1994.1535.

134. **Timpe, J. M., K. C. Verrill, and J. P. Trempe.** 2006. Effects of adeno-associated virus on adenovirus replication and gene expression during coinfection. Journal of Virology **80**:7807–7815.
135. **Tratschin, J. D., M. H. West, T. Sandbank, and B. J. Carter.** 1984. A human parvovirus, adeno-associated virus, as a eucaryotic vector: transient expression and encapsidation of the procaryotic gene for chloramphenicol acetyltransferase. Mol. Cell. Biol. **4**:2072–2081.
136. **Trempe, J. P., and B. J. Carter.** 1988. Regulation of adeno-associated virus gene expression in 293 cells: control of mRNA abundance and translation. Journal of Virology **62**:68–74.
137. **Urabe, M., Y. Hasumi, A. Kume, R. T. Surosky, G. J. Kurtzman, K. Tobita, and K. Ozawa.** 1999. Charged-to-alanine scanning mutagenesis of the N-terminal half of adeno-associated virus type 2 Rep78 protein. Journal of Virology **73**:2682–2693.
138. **Urcelay, E., P. Ward, S. M. Wiener, B. Safer, and R. M. Kotin.** 1995. Asymmetric replication in vitro from a human sequence element is dependent on adeno-associated virus Rep protein. Journal of Virology **69**:2038–2046.
139. **Vogel, R.** 2013. Viral and Cellular Components of AAV2 Replication Compartments. TOVJ **7**:98–120.
140. **Vogel, R., M. Seyffert, R. Strasser, A. P. de Oliveira, C. Dresch, D. L. Glauser, N. Jolinon, A. Salvetti, M. D. Weitzman, M. Ackermann, and C. Fraefel.** 2011. Adeno-Associated Virus Type 2 Modulates the Host DNA Damage Response Induced by Herpes Simplex Virus 1 during Coinfection. Journal of Virology **86**:143–155.
141. **Walz, C., A. Deprez, T. Dupressoir, M. Dürst, M. Rabreau, and J. R. Schlehofer.** 1997. Interaction of human papillomavirus type 16 and adeno-associated virus type 2 co-infecting human cervical epithelium. J. Gen. Virol. **78 (Pt 6)**:1441–1452.
142. **Wang, Y., S. M. Camp, M. Niwano, X. Shen, J. C. Bakowska, X. O. Breakefield, and P. D. Allen.** 2002. Herpes simplex virus type 1/adeno-associated virus rep(+) hybrid amplicon vector improves the stability of transgene expression in human cells by site-specific integration. Journal of Virology **76**:7150–7162.
143. **Ward, P., and K. I. Berns.** 1995. Minimum origin requirements for linear duplex AAV DNA replication in vitro. Virology **209**:692–695.

144. **Ward, P., and K. I. Berns.** 1996. In vitro replication of adeno-associated virus DNA: enhancement by extracts from adenovirus-infected HeLa cells. Journal of Virology **70**:4495–4501.
145. **Ward, P., P. Elias, and R. M. Linden.** 2003. Rescue of the adeno-associated virus genome from a plasmid vector: evidence for rescue by replication. Journal of Virology **77**:11480–11490.
146. **Ward, P., M. Falkenberg, P. Elias, M. Weitzman, and R. M. Linden.** 2001. Rep-dependent initiation of adeno-associated virus type 2 DNA replication by a herpes simplex virus type 1 replication complex in a reconstituted system. Journal of Virology **75**:10250–10258.
147. **Ward, P., E. Urcelay, R. Kotin, B. Safer, and K. I. Berns.** 1994. Adeno-associated virus DNA replication in vitro: activation by a maltose binding protein/Rep 68 fusion protein. Journal of Virology **68**:6029–6037.
148. **Ward, P., and C. E. Walsh.** 2012. Targeted integration of a rAAV vector into the AAVS1 region. Virology **433**:356–366.
149. **Weindler, F. W., and R. Heilbronn.** 1991. A subset of herpes simplex virus replication genes provides helper functions for productive adeno-associated virus replication. Journal of Virology **65**:2476–2483.
150. **Weitzman, M. D., K. J. Fisher, and J. M. Wilson.** 1996. Recruitment of wild-type and recombinant adeno-associated virus into adenovirus replication centers. Journal of Virology **70**:1845–1854.
151. **Weitzman, M. D., S. R. Kyostio, B. J. Carter, and R. A. Owens.** 1996. Interaction of wild-type and mutant adeno-associated virus (AAV) Rep proteins on AAV hairpin DNA. Journal of Virology **70**:2440–2448.
152. **Weitzman, M. D., S. R. Kyostio, R. M. Kotin, and R. A. Owens.** 1994. Adeno-associated virus (AAV) Rep proteins mediate complex formation between AAV DNA and its integration site in human DNA. Proceedings of the National Academy of Sciences of the United States of America **91**:5808–5812.
153. **Wilkinson, D. E., and S. K. Weller.** 2004. Recruitment of cellular recombination and repair proteins to sites of herpes simplex virus type 1 DNA replication is dependent on the composition of viral proteins within prereplicative sites and correlates with the induction of the DNA damage response. Journal of Virology **78**:4783–4796.
154. **Wilkinson, D. E., and S. K. Weller.** 2006. Herpes simplex virus type I disrupts the ATR-dependent DNA-damage response during lytic infection. Journal of Cell Science **119**:2695–2703.

155. **Wistuba, A., A. Kern, S. Weger, D. Grimm, and J. A. Kleinschmidt.** 1997. Subcellular compartmentalization of adeno-associated virus type 2 assembly. Journal of Virology **71**:1341–1352.
156. **Xiao, W., K. H. Warrington, JR., P. Hearing, J. Hughes, and N. Muzyczka.** 2002. Adenovirus-facilitated nuclear translocation of adeno-associated virus type 2. Journal of Virology **76**:11505–11517.
157. **Ylä-Herttuala, S.** 2012. Endgame: glybera finally recommended for approval as the first gene therapy drug in the European union. Mol. Ther. **20**:1831–1832.
158. **Young, S. M., JR., D. M. McCarty, N. Degtyareva, and R. J. Samulski.** 2000. Roles of adeno-associated virus Rep protein and human chromosome 19 in site-specific recombination. Journal of Virology **74**:3953–3966.
159. **Young, S. M., JR., and R. J. Samulski.** 2001. Adeno-associated virus (AAV) site-specific recombination does not require a Rep-dependent origin of replication within the AAV terminal repeat. Proceedings of the National Academy of Sciences of the United States of America **98**:13525–13530.
160. **Zarate-Perez, F., M. Bardelli, J. W. Burgner, M. Villamil-Jarauta, K. Das, D. Kekilli, J. Mansilla-Soto, R. M. Linden, C. R. Escalante, and C. Meyers.** 2012. The Interdomain Linker of AAV-2 Rep68 Is an Integral Part of Its Oligomerization Domain: Role of a Conserved SF3 Helicase Residue in Oligomerization. PLoS Pathog **8**:e1002764.
161. **Zarate-Perez, F., J. Mansilla-Soto, M. Bardelli, J. W. Burgner, M. Villamil-Jarauta, D. Kekilli, M. Samso, R. M. Linden, and C. R. Escalante.** 2012. Oligomeric Properties of Adeno-Associated Virus Rep68 Reflect Its Multifunctionality. Journal of Virology **87**:1232–1241.
162. **Zarate-Perez, F., J. Mansilla-Soto, M. Bardelli, J. W. Burgner, M. Villamil-Jarauta, D. Kekilli, M. Samso, R. M. Linden, and C. R. Escalante.** 2013. Oligomeric properties of adeno-associated virus Rep68 reflect its multifunctionality. J. Virol. **87**:1232–1241.

4. Specific Aims

1. The first aim was to characterize AAV2 Rep-mediated inhibition of HSV-1 replication, specifically to find out whether Rep is reducing HSV-1 gene expression and at what stage of the HSV-1 life-cycle the inhibition occurs.

2. The second aim was to identify the functional domains of Rep that are required and sufficient for inhibition of HSV-1 DNA replication.

3. The third aim was to discover the mechanism of Rep-mediated inhibition of HSV-1 replication. Two specific pathways were assessed: (i) the Rep-mediated induction of apoptosis and (ii) the Rep-induced activation of a DNA-damage response.

4. Aim four was to assess the role of the Rep-helicase activity in the inhibition of HSV-1 replication.

5. The fifth aim was to test whether the AAV2 Rep proteins are capable of binding to the HSV-1 dsDNA, thereby directing the inhibitory Rep-helicase activity towards the HSV-1 DNA and inhibiting the replication process.

6. The last aim was to characterize the phenotype of a novel Rep mutant RepD371Y.

5. Characterization of the AAV2 Rep-mediated Inhibition of HSV-1 DNA Replication

Original article

Inhibition of Herpes Simplex Virus Type 1 Replication by Adeno-Associated Virus Rep Proteins Depends on Their Combined DNA-Binding and ATPase/Helicase Activities

*Daniel L. Glauser,[1,2] *Michael Seyffert,[1] Regina Strasser,[1] Marco Franchini,[1] Andrea S. Laimbacher,[1] Christiane Dresch,[1] Anna Paula de Oliveira,[1] Rebecca Vogel,[1] Hildegard Büning,[3] Anna Salvetti,[4,5,6,7] Mathias Ackermann,[1] and Cornel Fraefel[1]

Institute of Virology, University of Zurich, Zurich, Switzerland[1];
Division of Virology, Department of Pathology, University of Cambridge, Cambridge, United Kingdom[2];
Clinic I for Internal Medicine and Center for Molecular Medicine, University of Cologne, Cologne, Germany[3];
INSERM U758, Lyon, France[4];
Ecole Normale Superieure de Lyon, Lyon, France[5];
IFR128 BioSciences Lyon-Gerland, Lyon, France[6];
Universite de Lyon, UCB-Lyon 1, Lyon, France[7]

JOURNAL OF VIROLOGY, Apr. 2010, p. 3808–3824

***D.L.G. and M.S. contributed equally to the work reported in this article**

Own Contribution

- Cloning of plasmids
- Western Blots
- Southern Blots
- Packaging assays
- Immuno-fluorescence
- Confocal microscopy
- Writing the manuscript
- Figure preparations

JOURNAL OF VIROLOGY, Apr. 2010, p. 3808–3824 Vol. 84, No. 8
0022-538X/10/$12.00 doi:10.1128/JVI.01503-09

Inhibition of Herpes Simplex Virus Type 1 Replication by Adeno-Associated Virus Rep Proteins Depends on Their Combined DNA-Binding and ATPase/Helicase Activities▽

Daniel L. Glauser,[1,2]† Michael Seyffert,[1]† Regina Strasser,[1] Marco Franchini,[1] Andrea S. Laimbacher,[1] Christiane Dresch,[1] Anna Paula de Oliveira,[1] Rebecca Vogel,[1] Hildegard Büning,[3] Anna Salvetti,[4,5,6,7] Mathias Ackermann,[1] and Cornel Fraefel[1]*

Institute of Virology, University of Zurich, Zurich, Switzerland[1]; Division of Virology, Department of Pathology, University of Cambridge, Cambridge, United Kingdom[2]; Clinic I for Internal Medicine and Center for Molecular Medicine, University of Cologne, Cologne, Germany[3]; INSERM U758, Lyon, France[4]; Ecole Normale Supérieure de Lyon, Lyon, France[5]; IFR128 BioSciences Lyon-Gerland, Lyon, France[6]; and Université de Lyon, UCB-Lyon 1, Lyon, France[7]

Received 20 July 2009/Accepted 18 January 2010

Adeno-associated virus (AAV) has previously been shown to inhibit the replication of its helper virus herpes simplex virus type 1 (HSV-1), and the inhibitory activity has been attributed to the expression of the AAV Rep proteins. In the present study, we assessed the Rep activities required for inhibition of HSV-1 replication using a panel of wild-type and mutant Rep proteins lacking defined domains and activities. We found that the inhibition of HSV-1 replication required Rep DNA-binding and ATPase/helicase activities but not endonuclease activity. The Rep activities required for inhibition of HSV-1 replication precisely coincided with the activities that were responsible for induction of cellular DNA damage and apoptosis, suggesting that these three processes are closely linked. Notably, the presence of Rep induced the hyperphosphorylation of a DNA damage marker, replication protein A (RPA), which has been reported not to be normally hyperphosphorylated during HSV-1 infection and to be sequestered away from HSV-1 replication compartments during infection. Finally, we demonstrate that the execution of apoptosis is not required for inhibition of HSV-1 replication and that the hyperphosphorylation of RPA *per se* is not inhibitory for HSV-1 replication, suggesting that these two processes are not directly responsible for the inhibition of HSV-1 replication by Rep.

Adeno-associated virus (AAV) is a widespread, nonpathogenic human parvovirus with a unique biphasic life cycle. In the absence of a helper virus, AAV establishes a latent infection in the host cell mediated either by site-specific integration of the viral genome into human chromosome 19 or by episomal persistence of circularized virus genomes (reviewed in reference 53). In the presence of helper viruses such as a herpesvirus, adenovirus (Ad), or papillomavirus, AAV is rescued from latency and undergoes lytic replication. The AAV genome is a single-stranded DNA (ssDNA) of 4,680 nucleotides, which is packaged into an icosahedral capsid with a diameter of 20 nm. The AAV genome harbors two open reading frames (ORFs), *rep* and *cap*, which are flanked by two inverted terminal repeats (ITRs) containing viral origins of DNA replication. The *cap* ORF is transcribed from the p40 promoter and encodes the capsid proteins VP1, VP2, and VP3, which differ in their N termini due to alternative start codons. The *rep* ORF encodes the Rep proteins, which are expressed in four different forms due to transcription from two different promoters, p5 and p19, and alternative splicing at an intron at the C-terminal end. The different Rep proteins are termed Rep40, Rep52, Rep68, and Rep78 according to their apparent molecular weight. The Rep proteins are involved in diverse processes in the viral life cycle, such as DNA replication, regulation of gene expression, genome packaging, and site-specific integration (reviewed in reference 56). The biochemical activities of Rep required for AAV DNA metabolism include site-specific DNA-binding and endonuclease activities, as well as non-site-specific ATPase/helicase activity. While the ATPase/helicase activity is in all four Rep proteins, the site-specific DNA-binding and endonuclease activities are present only in the large Rep proteins Rep68 and Rep78 (Fig. 1A) (18, 74, 88, 91). It has recently become clear that the Rep proteins also have a variety of effects on the host cell, the overall purpose of which likely is the creation of a cellular environment favorable for AAV replication. These effects of the Rep proteins on the cell include DNA damage, cell cycle arrest, apoptosis, and inhibition of signal transduction by the protein kinases PKA and PRKX. Rep78 can induce a complete S-phase arrest, which is mediated by the protein's ability to induce cellular DNA damage combined with its ability to bind to Cdc25A (5). The induction of DNA damage was postulated to require Rep endonuclease activity, while the interaction with Cdc25A was shown to be dependent on the zinc finger motifs in the C-terminal domain present in Rep52 and Rep78 (5) (Fig. 1A). The binding of Rep to Cdc25A prevents the latter from activating its substrates Cdk1 and Cdk2, resulting in the accumulation of hypophosphorylated (i.e., active) pRb, which in turn limits the cell's progression through S phase (5, 65). In addition and probably related to its ability to arrest the cell cycle, Rep has been shown

* Corresponding author. Mailing address: Institute of Virology, University of Zurich, Winterthurerstrasse 266a, CH-8057 Zurich, Switzerland. Phone: 41 44 6358713. Fax: 41 44 6358911. E-mail: cornelf@vetvir.uzh.ch.

† D.L.G. and M.S. contributed equally to the work reported in this article.

▽ Published ahead of print on 27 January 2010.

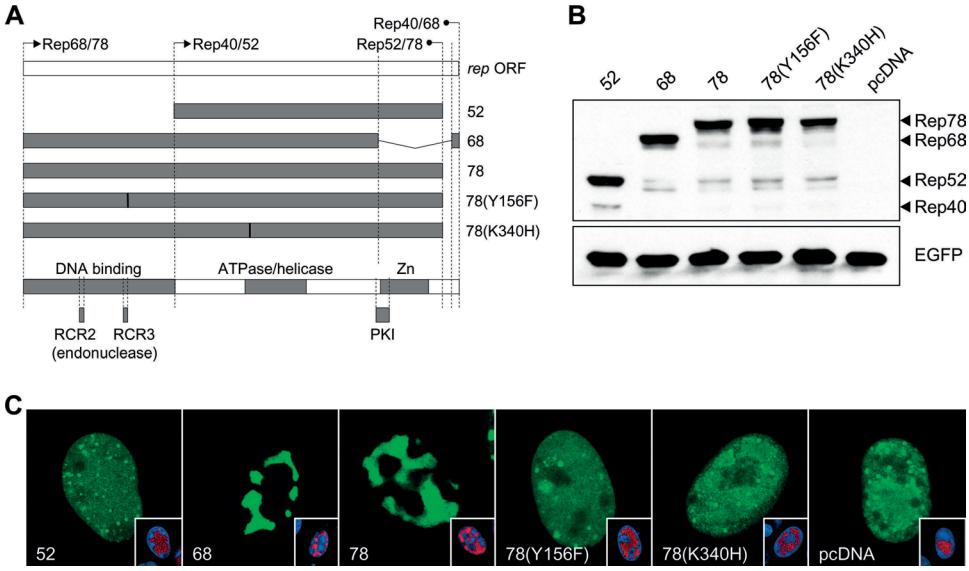


FIG. 1. (A) Rep proteins analyzed in this study. The open bar at the top represents the full-length AAV *rep* ORF, including the translation start sites of Rep68/78 and Rep40/52 (arrowheads) and the translation termination signals of Rep52/78 and Rep40/68 (circles). The gray boxes in the open bar at the bottom indicate regions of interest for this study, including the DNA-binding domain, the rolling-circle replication motifs 2 and 3 (RCR2 and RCR3) involved in the endonuclease activity, the ATPase/helicase domain, the PKI-like motif (PKI), and the zinc finger motifs (Zn). The gray bars in the middle show the Rep proteins expressed in this study, with mutations being indicated by black lines. Rep78 is the full-length Rep protein, while Rep52 lacks the N-terminal domain and thus DNA-binding and endonuclease activities, Rep68 lacks the C-terminal domain containing the PKI-like motif and the zinc finger motifs, Rep78(Y156F) contains a mutation in the RCR3 motif abolishing endonuclease activity, and Rep78(K340H) contains a mutation in the ATPase/helicase domain abolishing NTP-binding and thus helicase activity. (B) Rep expression levels. Vero cells were transfected with the pcDNA.Rep constructs or empty pcDNA vector together with pEGFP-N3, harvested 24 h later, and processed for Western blotting with a Rep-specific antibody. Detection of EGFP served as a transfection and loading control. (C) Evaluation of wild-type and mutant Rep proteins for their ability to replicate AAV DNA. Vero cells were transfected with pAAVlacO, pSV2-EYFP/lacI, and 0.1 μg of the pcDNA.Rep constructs or empty pcDNA vector. On the following day, the cells were infected with HSV-1 at an MOI of 10 PFU. At 14 h p.i., the cells were fixed and stained with an HSV-1 ICP8-specific MAb (red) and DAPI (4′,6′-diamidino-2-phenylindole) (blue). EYFP fluorescence is shown in green. Images were recorded by CLSM and show single *z* stacks of representative cells.

to induce p53-independent apoptosis via its DNA-binding and ATPase/helicase activities (66). Finally, the ability of Rep to inhibit the protein kinases PKA and PRKX, both members of the cyclic AMP (cAMP) signal transduction pathway, results in decreased expression of cAMP-responsive genes and contributes to Rep-mediated inhibition of Ad replication (16, 22, 23). Inhibition of PKA and PrKX was shown to depend on a PKI-like motif in the C-terminal domain present in Rep52 and Rep78 (Fig. 1A) (67).

Herpes simplex virus type 1 (HSV-1) is a complete helper virus for productive AAV replication (9). HSV-1 is a widespread human pathogen whose biphasic life cycle is characterized by lytic infection in epithelial cells of the mucosa and latent infection in the innervating sensory neurons. The HSV-1 genome is a 152-kb double-stranded DNA (dsDNA) and encodes approximately 80 gene products, which are expressed in a temporally regulated cascade comprising immediate-early (IE), early, and late phases (reviewed in reference 76). The HSV-1 helper factors for AAV replication act at two stages of the AAV life cycle, i.e., *rep* gene expression and DNA replication. The HSV-1 IE proteins ICP0, ICP4, and ICP22 synergistically transactivate *rep* expression, an effect which was shown to be particularly important for rescue of latent AAV (3, 28). The HSV-1 IE protein ICP27, in contrast, is inhibitory for AAV replication but essential for HSV-1 replication (3, 63). The HSV-1 helicase-primase complex (UL5/UL8/UL52), the ssDNA-binding protein ICP8 (UL29), and the DNA polymerase complex (UL30/UL42) are thought to become part of the AAV DNA replication complex, possibly through a direct interaction between ICP8 and Rep (3, 33, 71, 75, 80, 82). Although the helicase-primase complex and ICP8 are sufficient to support minimal levels of AAV DNA replication (82), the entire set of helper factors is required for the full helper activity (3).

The requirement of AAV for coinfection with a helper virus for productive replication inevitably leads to competition for cellular resources as well as for the helper factors themselves, which often are essential for both AAV and the helper virus. The fact that AAV inhibits the replication of its helper viruses Ad (10–12) and HSV (4, 31) suggests that it has developed strategies to influence this competition for its own advantage. In order to be successful, such strategies must limit the replication of the helper virus without affecting the synthesis of the helper factors required for AAV replication. In the case of HSV-1 as the helper virus, the helper factors are all expressed with IE or early kinetics preceding viral DNA replication (3, 81, 82). As expected for a successful strategy of helper virus inhibition, AAV limits mainly HSV-1 DNA replication and late gene expression, while IE and early gene expression is only marginally reduced (31). Similar observations were also made with Ad as the helper virus (77). Previous research from our laboratory has shown that AAV and HSV-1 replication proceeds in spatially separate replication compartments (RCs),

which recruit distinct sets of viral and cellular proteins. ICP8 was found in both HSV-1 and AAV RCs, although with differential staining patterns (31). It is therefore conceivable that the competition between HSV-1 and AAV RCs for ICP8 and possibly other HSV-1 replication factors contributes to inhibition of HSV-1 replication in coinfected cells. However, we and others have shown that the mere presence of AAV Rep protein in the absence of replicating AAV DNA is sufficient for inhibition of HSV-1 RC formation and HSV-1 oriS plasmid replication (31, 32). Similar observations were made for Ad in that Rep was shown to inhibit the maturation of Ad RCs (83). Rep-mediated inhibition of HSV-1 has also been observed during the production of chimeric AAV/HSV gene delivery vectors. Such vectors are based on helper virus-free HSV-1 amplicon vectors that incorporate the AAV ITRs flanking the transgene, as well as the AAV *rep* gene. HSV/AAV hybrid vectors combine the large transgene capacity of HSV-1 with the site-specific integration machinery of AAV and therefore support stable, long-term transgene expression (reviewed in reference 29). The presence of the *rep* gene on the hybrid vector genome, however, negatively affects vector replication, resulting in significantly reduced titers of vector stocks, and thus hampers efficient hybrid vector production (35).

To date, the molecular mechanisms underlying the inhibition of HSV-1 replication by AAV Rep protein remain poorly understood. We therefore further characterized Rep-mediated inhibition of HSV-1 replication in the present study. To this end, we investigated the effects of Rep on HSV-1 gene expression and DNA replication and assessed the Rep activities required for inhibition of HSV-1 replication using a panel of wild-type and mutant Rep proteins lacking defined domains and activities. Our data show that Rep mainly affects HSV-1 DNA replication and that the inhibitory effect of Rep depends on its DNA-binding and ATPase/helicase activities but not on its endonuclease activity. The Rep activities required for inhibition of HSV-1 replication precisely coincide with those required for the induction of cellular DNA damage and apoptosis, suggesting that these three processes are closely linked. Notably, the presence of Rep induced the hyperphosphorylation of a DNA damage marker, replication protein A (RPA), which has been reported not to be normally hyperphosphorylated during HSV-1 infection and to be sequestered away from HSV-1 replication compartments during infection. (84–86). Finally, we demonstrate that the execution of apoptosis is not required for inhibition of HSV-1 replication and that the hyperphosphorylation of RPA *per se* is not inhibitory for HSV-1 replication, suggesting that these two processes are not directly responsible for the inhibition of HSV-1 replication by Rep.

MATERIALS AND METHODS

Cell culture and viruses. Vero and Vero 2-2 cells (72) were maintained in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin G, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B. For culturing Vero 2-2 cells, 500 μg/ml G418 was included in addition.

HSV-1 strain F was grown and titers were determined in Vero cells. Briefly, confluent monolayers of Vero cells were infected with HSV-1 at a multiplicity of infection (MOI) of 0.1 PFU and incubated until the cytopathic effect (CPE) reached 100%. The cells were then lysed by three cycles of freeze and thawing and cellular debris removed by centrifugation for 10 min at 1,900 × *g*. The cleared lysate was titrated on Vero cells using the Spearman-Karber method. AAV type 2 (AAV2) was grown and titrated as described previously (46). UV inactivation of AAV2 with 254-nm UV light at a dose of 960 mJ/cm^2 was carried out in a UVC 500 UV cross-linker (Hoefer Pharmacia Biotech). The virus suspension was placed onto the lid of a 96-well tissue culture plate in order to form a layer of about 1 to 2 mm and kept on ice during the inactivation procedure. HSV-1 C12 is a recombinant HSV-1 strain SC16 containing a human cytomegalovirus (HCMV) IE1 enhancer/promoter-driven enhanced green fluorescent protein (EGFP) expression cassette in the US5 (gJ) locus and was kindly provided by S. Efstathiou (University of Cambridge, Cambridge, United Kingdom).

Plasmids. pcDNA3.1+ was purchased from Invitrogen and pEGFP-N3 from Clontech. pHSVGFP, an HSV-1 amplicon vector containing the EGFP-coding sequence under the control of the HSV-1 IE 4/5 promoter, was described previously (1). pAV2GFP, a recombinant AAV (rAAV) plasmid containing the EGFP-coding sequence under the control of the HCMV IE1 enhancer/promoter flanked by the AAV2 ITRs, was described previously (35). fHSVΔpacΔ27Δkn and pEBHICP27 together represent a replication-competent, packaging-defective HSV-1 genome and were described previously (64). Plasmids pCM-UL5, -UL8, -UL9, -UL29, -UL30, and -UL42, expressing the corresponding HSV-1 replication factors from the HCMV IE1 enhancer/promoter (34), were kindly provided by R. Heilbronn (Free University of Berlin, Berlin, Germany). Plasmid pD-UL52, expressing HSV-1 UL52 from the short eukaryotic initiation factor 1 α subunit (sEIFα) promoter, was described previously (3). pAAVlacO, an rAAV plasmid containing 40 *lac* operator (lacO) repeats flanked by the AAV2 ITRs, was described previously (25). Plasmid pRep, containing the AAV2 *rep* ORF under control of its native p5 and p19 promoters, was described previously (35). Plasmid pSV2-EYFP/lacI, expressing enhanced yellow fluorescent protein (EYFP) linked to *lac* repressor protein (LacI) (79), was kindly provided by D. L. Spector (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY). For the construction of plasmids pcDNA.Rep52, pcDNA.Rep68, pcDNARep78, and pcDNA.Rep78(K340H), the corresponding AAV2 *rep* ORFs were PCR amplified from plasmids pBP52, pBP68, pBP78, and pBP78^{K340H} (5, 65) (kindly provided by C. Berthet and P. Beard, ISREC, Lausanne, Switzerland) with primers containing BglII and XbaI restriction sites and inserted between the BamHI and XbaI restriction sites of pcDNA3.1+. For construction of pcDNA.Rep78(Y156F), the 359-bp SacII-BamHI *rep* fragment from plasmid pCL-HisRep68Y156F (27) containing the Y156F mutation was transferred into the *rep* ORF in plasmid pcDNA.Rep78. For construction of pcDNA.EGFP, the EcoRI-NotI fragment from pEGFP-N3 containing the ORF encoding enhanced green fluorescent protein (EGFP) was introduced between the EcoRI and NotI sites of pcDNA3.1+.

Antibodies. (i) Primary antibodies. The mouse anti-HSV-1 ICP8 monoclonal antibody (MAb) 7381 and the mouse anti-HSV-1 UL42 MAb Z1F11 were kindly provided by R. D. Everett (MRC Virology Unit, Glasgow, United Kingdom), the rabbit anti-HSV-1 ICP8 polyclonal antibody (PAb) 4-83 (41) by D. M. Knipe (Harvard Medical School, Boston, MA), the mouse anti-HSV-1 VP16 MAb LP1 (54) by A. Minson and H. Browne (University of Cambridge, Cambridge, United Kingdom), the rabbit anti-HSV-1 gC PAb R47 (17) by G. H. Cohen and R. J. Eisenberg (University of Pennsylvania, Philadelphia, PA), the rabbit anti-HSV-1 VP22 PAb AGV031 by G. Elliott (Imperial College London, London, United Kingdom), and the rabbit anti-AAV Rep PAb (78) by J. P. Trempe (Medical University of Ohio, Toledo, OH). The mouse anti-HSV-1 UL5 MAb 376, the mouse anti-HSV-1 UL8 MAb 817 (49, 55), the mouse anti-HSV-1 UL9 MAb 13924 (55), the mouse anti-HSV-1 UL30 MAb 13429 (48), and the mouse anti-HSV-1 UL52 MAb 14462 (48) were kindly provided by N. D. Stow (MRC Virology Unit, Glasgow, United Kingdom). The mouse anti-HSV-1 ICP4 MAb was purchased from Advanced Biotechnologies, the mouse anti-HSV-1 ICP8 MAb 10A3 from Abcam, the mouse anti-AAV Rep MAb clone 303.9 from Fitzgerald Industries International, the mouse anti-GFP MAb JL-8 from Clontech, the mouse antiactin MAb clone AC-47 from Sigma, the mouse anti-phospho-ATM S1981 MAb clone 10H11.E12 from Rockland Immunochemicals, the rabbit anti-phospho-RPA32 S4/S8 PAb BL647 from Bethyl Laboratories, and the mouse anti-phospho-H2AX S139 MAb clone JBW301 from Upstate (Millipore).

(ii) Secondary antibodies. Goat anti-mouse IgG(H+L)-Alexa Fluor 488 (AF488), goat anti-mouse IgG(H+L)-AF568, goat anti-mouse IgG(H+L)-AF594, goat anti-rabbit IgG(H+L)-AF488, goat anti-rabbit IgG(H+L)-AF568, and F(ab′)$_2$ fragment of goat anti-rabbit IgG(H+L)-AF594 were purchased from Molecular Probes (Invitrogen), rabbit anti-mouse IgG(whole molecule)-peroxidase from Sigma, and goat anti-rabbit IgG(H+L)-horseradish peroxidase (HRP) from Southern Biotech.

Transfection. Cells seeded on the previous day were transfected with Lipofectamine and Plus reagents (Invitrogen) according to the manufacturer's protocol, except for the data presented in Fig. 5, for which Lipofectamine2000 (Invitrogen) was used. Transfections were carried out in three different plate

TABLE 1. Summary of Rep constructs and their activities

Rep protein	DNA binding	Endonuclease on:		ATPase/helicase	C-terminal domain	Replication of AAV	Inhibition of HSV-1	Apoptosis	DNA damage
		ssDNA	dsDNA						
Rep52	−	−	−	+	+	−	−	−	−
Rep68	+	+	+	+	−	+	+	+	+
Rep78	+	+	+	+	+	+	+	+	+
Rep78(Y156F)	+	−	−	+	+	−	+	+	+
Rep78(K340H)	+	+	−	−	+	−	−	+/−	−

formats: 6-cm plates (Southern blot analysis), 12-well plates (Western blot analysis), and 24-well plates (pHSVGFP packaging, HSV-1 C12 replication, and annexin V and immunofluorescence staining). The relative amounts of DNA used for transfection of different plate formats were as follows: 6-cm plate, 100%; 12-well plate, 20%; and 24-well plate, 10%. For easier comparability of DNA amounts between the different assays, the DNA amounts indicated throughout correspond to values normalized to the 6-cm format.

Western blot analysis. For the results shown in Fig. 1B, Vero cells were transfected with 0.5 μg of the individual pcDNA.Rep plasmids or empty pcDNA vector together with 0.5 μg pEGFP-N3. For the results presented in Fig. 2D, Vero 2-2 cells were transfected with 0.5 μg pHSVGFP, 2 μg fHSVΔpacΔ27Δkn, and 0.2 μg pEBHICP27 together with the indicated amounts of pcDNA.Rep plasmids or empty pcDNA vector. For the results presented in Fig. 3A, Vero cells were transfected with 0.5 μg pHSVGFP, 0.2 μg each of pCM-UL5/8/9/29/30/42 plasmids, and 0.4 μg of pD-UL52 together with the indicated amounts of pcDNA.Rep plasmids or empty pcDNA vector. At 48 h after transfection, the cells were lysed and processed for Western blot analysis as described previously (31). Primary antibodies were used at the following dilutions: mouse anti-AAV Rep MAb clone 303.9, 1:200; mouse anti-GFP MAb JL-8, 1:8,000; mouse anti-HSV-1 ICP4 MAb, 1:10,000; rabbit anti-HSV-1 ICP8 PAb 4-83, 1:5,000; mouse anti-HSV-1 ICP8 MAb 10A3, 1:1,000; mouse anti-HSV-1 UL5 MAb 376, 1:1,000; mouse anti-HSV-1 UL8 MAb 817, 1:500; mouse anti-HSV-1 UL9 MAb 13924, 1:500; mouse anti-HSV-1 UL30 MAb 13429, 1:500; mouse anti-HSV-1 UL52 MAb 14462, 1:200; mouse anti-HSV-1 UL42 MAb Z1F11, 1:1,000; mouse anti-HSV-1 VP16 MAb LP1, 1:5,000; rabbit anti-HSV-1 VP22 AGV031, 1:10,000; rabbit anti-HSV-1 gC PAb R47, 1:10,000; and mouse antiactin MAb clone AC-47, 1:10,000. Secondary antibodies were used at the following dilutions: rabbit anti-mouse IgG(whole molecule)-peroxidase, 1:10,000; goat anti-rabbit IgG(H+L)-HRP, 1:10,000.

Live visualization assay for AAV DNA replication. The live visualization assay for AAV DNA replication has been described previously (25). Briefly, Vero cells grown on coverslips were transfected with 0.5 μg of the pAAVlacO replicon, 0.1 μg of the EYFP-LacI-expressing plasmid pSV2-EYFP/lacI, and 0.1 μg of the pcDNA.Rep plasmids or the empty pcDNA vector. On the following day, the cells were infected with HSV-1 at an MOI of 10 PFU to provide helper functions for AAV replication. The cells were fixed at 14 h postinfection (p.i.) and processed for immunofluorescence staining.

Immunofluorescence. Immunofluorescence staining was performed as described previously (31). The primary antibodies were used at the following dilutions: mouse anti-HSV-1 ICP8 MAb 7381, 1:1,000; mouse anti-AAV Rep MAb, 1:10; rabbit anti-AAV Rep PAb, 1:400; rabbit anti-phospho-RPA32 S4/S8 PAb, 1:100; mouse anti-phospho-ATM S1981 MAb, 1:100; and mouse anti-phospho-H2AX S139 MAb, 1:100. Alexa Fluor-conjugated secondary antibodies were diluted 1:200 to 1:1,000. Confocal laser scanning microscopy (CLSM) was performed as described previously (21).

Packaging of HSV-1 amplicons. Packaging of the HSV-1 amplicon pHSVGFP was done essentially as described previously (64). Briefly, Vero 2-2 cells were transfected with 0.5 μg of pHSVGFP, 2 μg of fHSVΔpacΔ27Δkn, and 0.2 μg of pEBHICP27 together with the indicated amounts of pcDNA.Rep plasmids or empty pcDNA vector. Three days later, cells were harvested and HSV-1 amplicon particles released from cells by three cycles of freezing and thawing. Cellular debris was removed by centrifugation at 1,900 × *g* for 5 min. The cleared supernatant was titrated by infection of Vero cells and enumeration of $EGFP^+$ cells 48 h p.i. by flow cytometry on a FACSCalibur (BD Biosciences).

Southern blot analysis. For the results presented in Fig. 2E and F, Vero 2-2 cells were transfected with 0.5 μg of pHSVGFP or pAV2GFP, 2 μg of fHSVΔpacΔ27Δkn, and 0.2 μg of pEBHICP27 together with the indicated amounts of pcDNA.Rep78 or empty pcDNA vector. For the results presented in Fig. 3B, Vero 2-2 cells were transfected with 0.5 μg of pHSVGFP, 1 μg of fHSVΔpacΔ27Δkn, 0.1 μg of pEBHICP27, and 0.2 μg each of pCM-UL5/8/9/29/30/42 and pD-UL52 plasmids together with the indicated amounts of pcDNA.Rep78 or empty pcDNA vector. For the results presented in Fig. 3C, Vero 2-2 cells were transfected with 0.5 μg of pHSVGFP, 0.1 μg of pEBHICP27, and 0.4 μg each of pCM-UL5/8/9/29/30/42 and pD-UL52 plasmids together with the indicated amounts of pcDNA.Rep78 or empty pcDNA vector. Three days later, the cells were harvested and extrachromosomal DNA extracted by the procedure described by Hirt (36). The DNA was digested as indicated in the text, separated on 1% agarose gels, and transferred to positively charged nylon membranes (Hybond N+; Amersham). Hybridization with a digoxigenin (DIG)-labeled probe specific for the EGFP-coding sequence and immunological detection using an alkaline phosphatase-conjugated anti-DIG antibody and chemiluminescence substrate (CDP Star) were performed as described by the supplier (Roche). The DIG-labeled probe was produced by PCR amplification of the ORF encoding EGFP from plasmid pEGFP-N3 using the PCR DIG probe synthesis kit (Roche). The PCR product was purified with the QIAquick PCR purification kit (Qiagen) before being used for hybridization.

Treatment with HU, CPT, and UV-AAV. Hydroxyurea (HU) and camptothecin (CPT) were purchased from Sigma and stock solutions prepared in H_2O and dimethyl sulfoxide (DMSO), respectively. Vero cells were treated for the indicated times with 2.5 mM HU, 1 μM CPT (or DMSO as a control), and 10^4 or 5×10^4 genome-containing particles (gcp) of UV-inactivated AAV (UV-AAV). The cells were then either processed for immunofluorescence staining (see Fig. 6A to C) or washed three times with phosphate-buffered saline (PBS) followed by infection with HSV-1 C12 at an MOI of 5 PFU (see Fig. 6E). When the CPE reached 100% (30 h p.i.), cells were harvested and virus particles released from cells by three cycles of freezing and thawing. Cellular debris was removed by centrifugation at 1,900 × *g* for 5 min. The cleared supernatant was titrated by infection of Vero cells in the presence of 100 μg/ml phosphonoacetic acid (PAA) and enumeration of $EGFP^+$ cells at 24 h p.i. by flow cytometry on a FACSCalibur (BD Biosciences).

Treatment with caspase inhibitors. The broad-spectrum caspase inhibitors Z-VAD(OMe)-FMK (caspase inhibitor I) and Boc-D-FMK (caspase inhibitor III) were purchased from Calbiochem (Merck). Stock solutions were prepared in DMSO and added to cell culture media at final concentrations of 100 μM. Control cells were treated with the solvent DMSO alone. Every 24 h, half of the cell culture medium was removed and replaced by medium containing fresh caspase inhibitors or DMSO.

Annexin V staining. Vero cells were transfected with 0.25 μg pEGFP-N3 together with 0.5 or 1 μg pcDNA.Rep plasmids or empty pcDNA vector as indicated in Results. Annexin V staining was performed using the annexin V-Cy5 Apoptosis Detection Kit from Abcam according to the manufacturer's manual. The cells were analyzed by flow cytometry on a FACSCalibur (BD Biosciences) with filters specific for EGFP (transfected cells) and Cy5 (annexin V^+ cells).

RESULTS

Expression of wild-type and mutant AAV Rep proteins. For high-level, constitutive expression of Rep proteins, the corresponding ORFs were placed under control of the HCMV IE1 enhancer/promoter in the pcDNA3.1+ expression vector. A map of the Rep proteins used in this study is shown in Fig. 1A, and their activities are summarized in Table 1. Briefly, we compared the full-length Rep78 protein with (i) Rep52, which lacks the N-terminal site-specific DNA-binding and endonuclease activities (60); (ii) Rep68, which lacks the C-terminal PKI-like and zinc finger motifs (22, 37); (iii) Rep78(Y156F),

which contains a mutation in rolling-circle replication motif 3 (RCR3), thereby abolishing site-specific endonuclease activity (20, 73); and (iv) Rep78(K340H), which contains a mutation in the consensus nucleoside triphosphate (NTP)-binding site, thereby abolishing ATPase/helicase activity (13, 43). The loss of ATPase/helicase activity in the K340H mutant results in a loss of endonuclease activity on dsDNA substrates, because endonuclease activity on dsDNA requires prior unwinding via the helicase activity (50, 60). Of note also is that the K340H mutant still retains endonuclease activity on ssDNA substrates, while the Y156F mutant lacks endonuclease activity on both ssDNA and dsDNA substrates (20, 73) (Table 1).

To assess the expression levels from the different constructs, Vero cells were transfected with the Rep-encoding plasmids together with a plasmid expressing EGFP, which served as transfection and loading control. Western blotting with Rep- and EGFP-specific antibodies showed that all Rep proteins had the expected sizes and were expressed at comparable levels (Fig. 1B). To ascertain the functionality of the expressed Rep proteins, we tested their ability to support AAV DNA replication. To this end, we employed a previously described live cell visualization assay for AAV replication, in which AAV RCs are visualized by the binding of an EYFP-LacI fusion protein to lacO repeats present in a recombinant AAV (rAAV) genome (25). The rAAV genome and the plasmid encoding the EYFP-LacI fusion protein were transfected together with the individual Rep-encoding plasmids, while the helper factors for AAV replication were provided by infection with HSV-1. Transfected cells were identified by EYFP-LacI expression, while HSV-1-infected cells were identified by staining for HSV-1 ICP8, which localizes to HSV-1 and AAV RCs. AAV DNA replication is known to require Rep DNA-binding, endonuclease, and ATPase/helicase activities (7, 8, 38, 39, 50). In line with this, AAV RCs were observed only in cells transfected with the Rep68 and Rep78 constructs, while Rep52, Rep78(Y156F), and Rep78(K340H), as well as the empty vector, did not support AAV DNA replication (Fig. 1C).

Taken together, these results demonstrate that the different Rep constructs used in this study are expressed at comparable levels and meet the expected profile regarding their ability to replicate AAV DNA (summarized in Table 1).

AAV Rep inhibits HSV-1 gene expression and DNA replication. Although Rep-mediated inhibition of HSV-1 replication has previously been reported (31, 32, 35), the molecular mechanisms underlying theses observations remain unclear. We therefore set out to further characterize the effect of Rep on the replication of HSV-1. As a global measure of HSV-1 replication efficiency, we employed a previously described HSV-1 amplicon packaging assay (1, 59, 64). Briefly, Vero 2-2 cells, which express HSV-1 ICP27 from its native promoter (72), were transfected with an HSV-1 amplicon plasmid encoding EGFP, pHSVGFP, together with an HSV-1 helper bacterial artificial chromosome (BAC), fHSVΔpacΔ27Δkn, and an ICP27 expressing plasmid, pEBHICP27. The HSV-1 amplicon plasmid pHSVGFP contains the HSV-1 oriS, the HSV-1 cleavage/packaging signal (*pac*), and an EGFP expression cassette. It is replicated and packaged into HSV-1 particles in the presence of HSV-1 helper functions, and titers of vectors stocks can readily be determined with help of the EGFP reporter transgene (1, 59). The HSV-1 helper BAC fHSVΔpacΔ27Δkn consists of an HSV-1 genome with the ICP27 gene and *pac* deleted (the nonessential $\gamma_1$34.5 gene is also deleted due to deletion of *pac*). In the presence of *trans*-expressed ICP27, the HSV-1 helper BAC represents a replication-competent, packaging-defective HSV-1 genome providing helper functions for replication and packaging of HSV-1 amplicon plasmids (59, 64). Although Vero 2-2 cells express ICP27, the cotransfection of an ICP27-expressing plasmid is required for high titers of HSV-1 amplicon vector stocks (64). Packaged HSV-1 amplicon stocks were harvested at 72 h after transfection and the titers determined by enumerating $EGFP^+$ cells upon infection of Vero cells. By providing the helper functions from a packaging-defective HSV-1 genome, we ensured that replication of the HSV-1 amplicon could occur only in transfected cells and that HSV-1 infection could not spread to neighboring, untransfected cells. The effects of Rep on HSV-1 replication could therefore readily be assessed by cotransfection of Rep encoding plasmids together with the HSV-1 amplicon and HSV-1 helper DNA. As shown in Fig. 2A, the cotransfection of increasing amounts of the construct encoding the full-length Rep protein, Rep78, led to a pronounced, dose-dependent inhibition of HSV-1 replication, demonstrating that the HSV-1 amplicon packaging assay provides a sensitive means to assess the effects of Rep on HSV-1 replication efficiency.

We have previously demonstrated that AAV-mediated inhibition of HSV-1 replication in the context of AAV/HSV-1 coinfection occurs mainly at the stage of DNA replication (31). In order to find out if this also holds true for inhibition of HSV-1 by expression of Rep78, we assessed the amounts of replicated HSV-1 amplicon DNA by Southern blotting. Cells transfected with increasing amounts of the Rep78 construct together with HSV-1 amplicon DNA (pHSVGFP) and HSV-1 helper DNA were harvested 72 h later for extraction of extrachromosomal DNA. To distinguish between transfected and newly synthesized HSV-1 amplicon DNA, the DNA was digested with DpnI, which selectively cleaves the transfected DNA of bacterial origin (i.e., Dam-methylated DNA). The DNA was also cleaved with HindIII, which cuts once on pHSVGFP and therefore reduces the concatemeric replication products into linear monomers. HSV-1 amplicon DNA was detected with a probe specific for the EGFP-coding sequence. Figure 2E shows that transfection of the Rep78 plasmid led to a dose-dependent reduction of the DpnI-resistant HSV-1 amplicon replication products, demonstrating that Rep inhibits HSV-1 DNA replication.

Inhibited HSV-1 DNA replication can theoretically result either from blocks at the stages of IE or early gene expression or from a block of DNA replication itself. To determine which of the above possibilities holds true for our experimental system, we analyzed HSV-1 gene expression in the presence of Rep78. Cells transfected with increasing amounts of the Rep78 construct together with HSV-1 amplicon DNA (pHSVGFP) and HSV-1 helper DNA were harvested 48 h later and subjected to Western analysis with antibodies specific for HSV-1 proteins of all kinetic classes. As shown in Fig. 2D, expression of Rep78 resulted in a dose-dependent inhibition of the levels of the IE protein ICP4; the early proteins UL9, ICP8, UL5, and UL42; and the late proteins VP16, VP22, and glycoprotein C (gC). Of note, the inhibitory effect on the late proteins was more pronounced than that on the IE and early proteins. (We

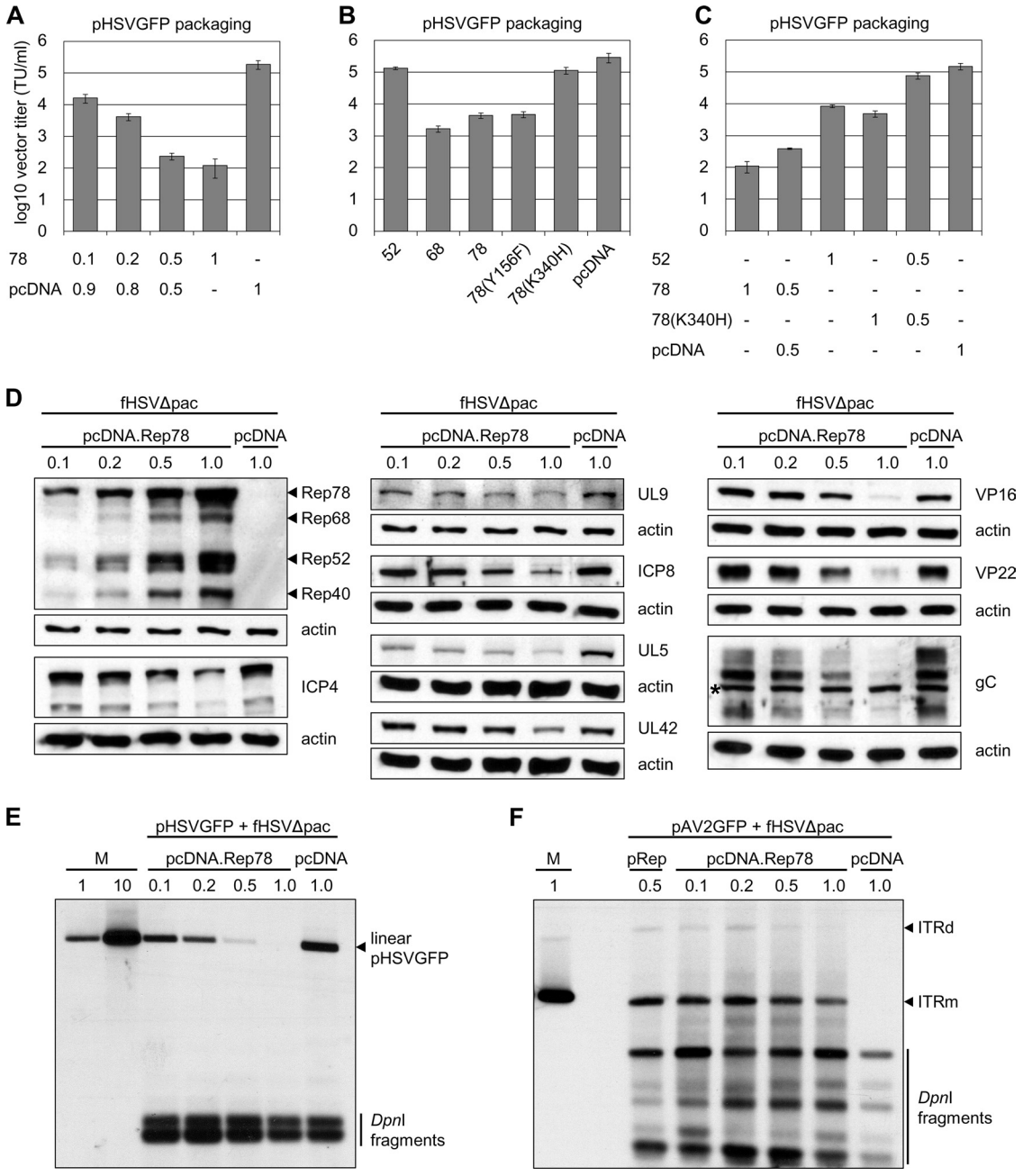


FIG. 2. (A) Effects of Rep78 on HSV-1 productivity. Vero 2-2 cells were transfected with pHSVGFP, fHSVΔpacΔ27Δkn, pEBHICP27, and the indicated amounts (in μg) of pcDNA.Rep78 or empty pcDNA vector. The pHSVGFP amplicon vectors were harvested 72 h later and titrated on Vero cells. (B) Screening of Rep proteins for inhibitory activity on HSV-1 replication. Vero 2-2 cells were transfected with pHSVGFP, fHSVΔpacΔ27Δkn, pEBHICP27, and 0.5 μg each of the pcDNA.Rep constructs or empty pcDNA vector. The pHSVGFP amplicon vectors were harvested 72 h later and titrated on Vero cells. (C) *trans*-complementation of Rep DNA-binding and ATPase/helicase activities. Vero 2-2 cells were transfected with pHSVGFP, fHSVΔpacΔ27Δkn, pEBHICP27, and the indicated amounts (in μg) of the pcDNA.Rep constructs or empty pcDNA vector. The pHSVGFP amplicon vectors were harvested 72 h later and titrated on Vero cells. The data in panels A to C are shown as means ± standard deviations (SD) from triplicate experiments. (D) Effects of Rep on HSV-1 protein levels. Vero 2-2 cells were transfected with pHSVGFP, fHSVΔpacΔ27Δkn, pEBHICP27, and the indicated amounts (in μg) of pcDNA.Rep78 or empty pcDNA vector. At 48 h after transfection, the cells were lysed and analyzed by Western blotting with antibodies specific for AAV Rep and HSV-1 ICP4, UL9, ICP8, UL5, UL42, VP16, VP22, and gC. Detection of actin served as a loading control. The asterisk indicates an unspecific band. (E) Effects of Rep78 on HSV-1 DNA replication. Vero 2-2 cells were transfected as described for panel D. At 72 h after transfection, extrachromosomal DNA was extracted, digested with DpnI and HindIII, and analyzed by Southern blotting with a DIG-labeled probe specific for the EGFP-coding sequence on pHSVGFP. The linearized pHSVGFP replication products (DpnI resistant) and the DpnI fragments of the transfected pHSVGFP plasmid (DpnI sensitive) are indicated. One or 10 ng of linearized pHSVGFP plasmid was loaded as a positive control and size marker (M). (F) Effects of Rep on AAV DNA replication. Vero 2-2 cells were transfected with pAV2GFP, fHSVΔpacΔ27Δkn, pEBHICP27, and the indicated amounts (in μg) of pRep, pcDNA.Rep78, or empty pcDNA vector. At 72 h after transfection, extrachromosomal DNA was extracted, digested with DpnI, and analyzed by Southern blotting with a DIG-labeled probe specific for the EGFP-coding sequence on rAV2GFP. Rescued monomeric (ITRm) and dimeric (ITRd) rAV2GFP replication intermediates (DpnI resistant) and the DpnI fragments of the transfected pAV2GFP plasmid (DpnI sensitive) are indicated. One nanogram of the BglII-excised ITR cassette from pAV2GFP was loaded as a positive control and size marker (M).

also attempted to detect UL8, UL52, and UL30, but the levels expressed in HSV-1 helper BAC-transfected cells were too low for detection by Western blotting.)

To assess if the observed inhibition of HSV-1 gene expression and DNA replication is specific for HSV-1 and not due to general cytotoxicity of Rep, we assessed whether the amounts of Rep78 plasmid used were compatible with AAV DNA replication. General cytotoxic effects of Rep would be expected to inhibit both HSV-1 and AAV DNA replication. To this end, we performed an replication assay equivalent to that shown in Fig. 2E, except that an rAAV plasmid (pAV2GFP) was used instead of the HSV-1 amplicon. AAV DNA replication from pAV2GFP results in the rescue of DpnI-resistant mono- and multimeric ITR cassettes (i.e., rAAV replication intermediates). rAAV DNA was detected with a probe specific for the EGFP-coding sequence. Transfection of a plasmid encoding all four Rep proteins under control of the native p5 and p19 promoters, pRep, served as a control for physiological Rep levels. The blot in Fig. 2F shows that all the amounts of Rep78 plasmid used supported AAV DNA replication, while no AAV DNA replication was observed in the absence of Rep. Specifically, the levels of the AAV replication intermediates were comparable between pRep and 0.1 to 0.5 μg of pcDNA.Rep78, while the levels were slightly reduced with 1 μg of pcDNA.Rep78, indicating that very high Rep levels are inhibitory to AAV replication (Fig. 2F).

The seven essential HSV-1 replication factors, UL9, ICP8, UL5/8/52, and UL30/42, are all expressed with early kinetics (81). We therefore aimed to find out if the observed inhibition of the HSV-1 early gene expression (Fig. 2D), in particular the expression of the replication factors, was responsible for the observed inhibition of HSV-1 DNA replication or if, alternatively, Rep rather had a direct inhibitory effect on HSV-1 DNA replication. If the former was the case, Rep would not be expected to inhibit HSV-1 DNA replication if abundant levels of HSV-1 replication factors were provided by overexpression from constitutive promoters. If, however, Rep acted directly on HSV-1 DNA replication, inhibition of HSV-1 DNA replication would be expected even in the presence of overexpressed replication factors. We first tested the effect of Rep78 on the levels of the seven HSV-1 replication factors when expressed from the HCMV IE1 enhancer/promoter (UL9, ICP8, UL5, UL8, UL30, and UL42) or the short eukaryotic initiation factor 1 α subunit (sEIFα) promoter (UL52). As shown in Fig. 3A, Rep78 had no significant inhibitory effect on the levels of the HSV-1 replication factors expressed from constitutive promoters. We next assessed the effect of Rep78 on pHSVGFP amplicon replication mediated by HSV-1 helper BAC and overexpressed HSV-1 replication factors (Fig. 3B) or by overexpressed HSV-1 replication factors alone (Fig. 3C). In either situation, Rep78 significantly inhibited the replication of the HSV-1 amplicon, demonstrating that Rep inhibits HSV-1 DNA replication even in the presence of abundant HSV-1 replication factors.

Taken together, these findings show that the expression of Rep78 from the HCMV IE1 enhancer/promoter strongly inhibits HSV-1 productivity (Fig. 2A), HSV-1 gene expression (Fig. 2D), and HSV-1 DNA replication (Fig. 2E). The inhibitory effects of Rep78 on HSV-1 appeared to be specific and not due to general cytotoxicity, since the Rep levels employed were compatible with AAV DNA replication (Fig. 2F). Furthermore, the data show that the inhibition of HSV-1 DNA replication by Rep was not due to the reduction of the expression of the HSV-1 replication factors but presumably was caused by a direct inhibitory effect of Rep on HSV-1 DNA replication (Fig. 3). The finding that Rep levels compatible with efficient AAV DNA replication are strongly inhibitory for HSV-1 DNA replication (Fig. 2E and F) further corroborates this notion, since both AAV and HSV-1 DNA replication depend on the HSV-1 replication factors (3).

Rep DNA-binding and ATPase/helicase activities are required for inhibition of HSV-1. We next aimed to identify which activities of Rep78 were required for the observed inhibition of HSV-1 replication. For this, all Rep constructs presented in Fig. 1 were screened for their ability to inhibit HSV-1 replication. This time we transfected 0.5 μg of the Rep plasmids, since this amount was sufficient for considerable inhibition of HSV-1 replication by Rep78 (Fig. 2A and E). As shown in Fig. 2B, Rep68, Rep78, and Rep78(Y156F) all inhibited HSV-1 replication at comparable levels, while almost no inhibition was observed in the presence of Rep52 and Rep78 (K340H). The lack of inhibitory activity of Rep52 and Rep78(K340H) demonstrates that DNA-binding and ATPase/helicase activities are essential for inhibition of HSV-1 replication. The finding that Rep78(Y156F) and Rep68 inhibit HSV-1 replication allows to conclude that endonuclease activity and the C-terminal domain containing the PKI-like and the zinc finger motifs are not required for inhibition of HSV-1.

We then asked if Rep DNA-binding and ATPase/helicase activities need to be present on the same Rep molecule or if *trans*-complementation of DNA-binding and ATPase/helicase activities would restore the inhibitory effect. Although Rep78 (K340) has a dominant-negative effect on the helicase activity of wild-type Rep78, it does not have any dominant-negative effect on Rep52 helicase activity (74). Transfection of cells with Rep78(K340H), which has site-specific DNA-binding activity but no ATPase/helicase activity, together with Rep52, which has ATPase/helicase activity but no site-specific DNA-binding activity, therefore allows us to assess if Rep DNA-binding and ATPase/helicase activities act separately or if the ATPase/helicase activity of Rep needs to be targeted to a dsDNA substrate via the site-specific DNA-binding domain. As shown in Fig. 2C, cotransfection of Rep78(K340H) with Rep52 did not restore the inhibitory effect, suggesting that the two activities need to be present in *cis*.

Taken together, these results demonstrate that Rep-mediated inhibition of HSV-1 replication does not depend on the endonuclease activity or the C-terminal domain but requires DNA-binding and ATPase/helicase activities on the same Rep molecule (summarized in Table 1).

The activities of Rep required for inhibition of HSV-1 replication coincide with those required for induction of apoptosis. Rep proteins have previously been described to induce apoptosis in HL-60 and NT2 cells, which are a p53-null promyeloid cell line and a p53-containing embryonal carcinoma cell line, respectively (66). The induction of apoptosis has been shown to be mediated by the DNA-binding and ATPase/helicase activities of Rep (66), notably the same activities which are required for inhibition of HSV-1 replication.

We therefore first wanted to determine if Rep expression is also able to induce apoptosis in Vero cells. In the following

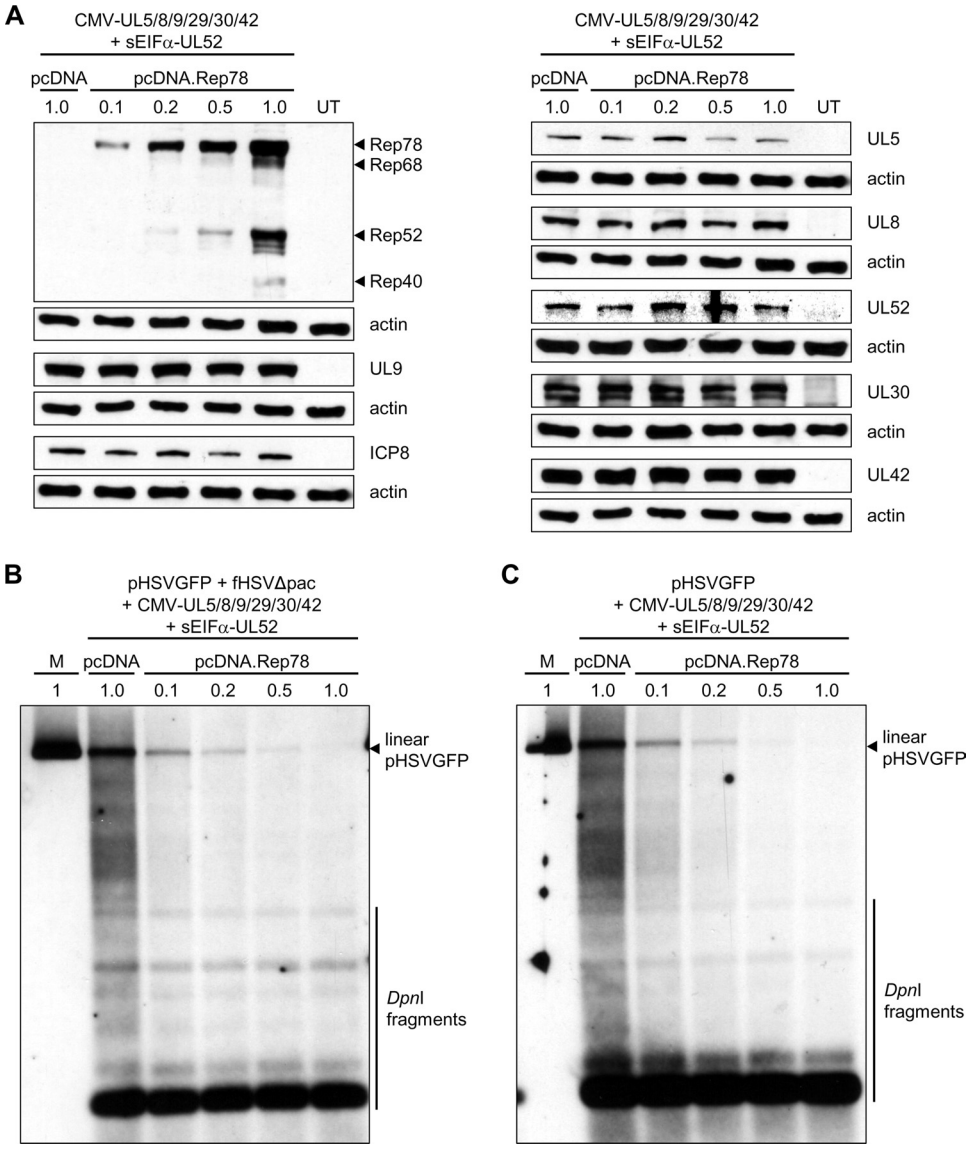


FIG. 3. (A) Effects of Rep on levels of HSV-1 replication factors expressed from constitutive promoters. Vero cells were transfected with pCM-UL5, -UL8, -UL9, -UL29, -UL30, -UL42, pD-UL52, and the indicated amounts (in μg) of pcDNA.Rep78 or empty pcDNA vector. Untransfected cells were loaded as a control (UT). At 48 h after transfection, the cells were lysed and analyzed by Western blotting with antibodies specific for AAV Rep and HSV-1 UL9, ICP8, UL5, UL8, UL52, UL30, and UL42. Detection of actin served as a loading control. (B) Effects of Rep on pHSVGFP amplicon replication mediated by HSV-1 helper BAC and overexpressed HSV-1 replication factors. Vero 2-2 cells were transfected with pHSVGFP, fHSVΔpacΔ27Δkn, pEBHICP27, pCM-UL5, -UL8, -UL9, -UL29, -UL30, -UL42, pD-UL52, and the indicated amounts (in μg) of pcDNA.Rep78 or empty pcDNA vector. At 72 h after transfection, extrachromosomal DNA was extracted, digested with DpnI and HindIII, and analyzed by Southern blotting with a DIG-labeled probe specific for the EGFP-coding sequence on pHSVGFP. The linearized pHSVGFP replication products (DpnI resistant) and the DpnI fragments of the transfected pHSVGFP plasmid (DpnI sensitive) are indicated. One nanogram of linearized pHSVGFP plasmid was loaded as a positive control and size marker (M). (C) Effects of Rep on pHSVGFP amplicon replication mediated by overexpressed HSV-1 replication factors. Vero 2-2 cells were transfected with pHSVGFP, pEBHICP27, pCM-UL5, -UL8, -UL9, -UL29, -UL30, -UL42, pD-UL52, and the indicated amounts (in μg) of pcDNA.Rep78 or empty pcDNA vector. Southern blotting was performed as described for panel B.

experiments, unless otherwise stated, cells were transfected with 0.5 μg of the Rep constructs. In a first experiment, we assessed the time course of Rep-mediated apoptosis. For this, Vero cells were transfected with the Rep78 construct or the empty vector, together with a plasmid expressing EGFP (pEGFP-N3), and analyzed 24, 48, and 72 h later by staining with annexin V followed by flow cytometry. Transfected cells were identified by EGFP fluorescence. The results shown in Fig. 4A show that Rep78 is able to induce apoptosis in Vero cells starting from 48 h after transfection. We next assessed if the observed induction of apoptosis in Vero cells also depends on the DNA-binding and ATPase/helicase activities of Rep, as previously reported for HL-60 and NT2 cells (66). This was indeed the case, as apoptosis was readily induced by Rep68,

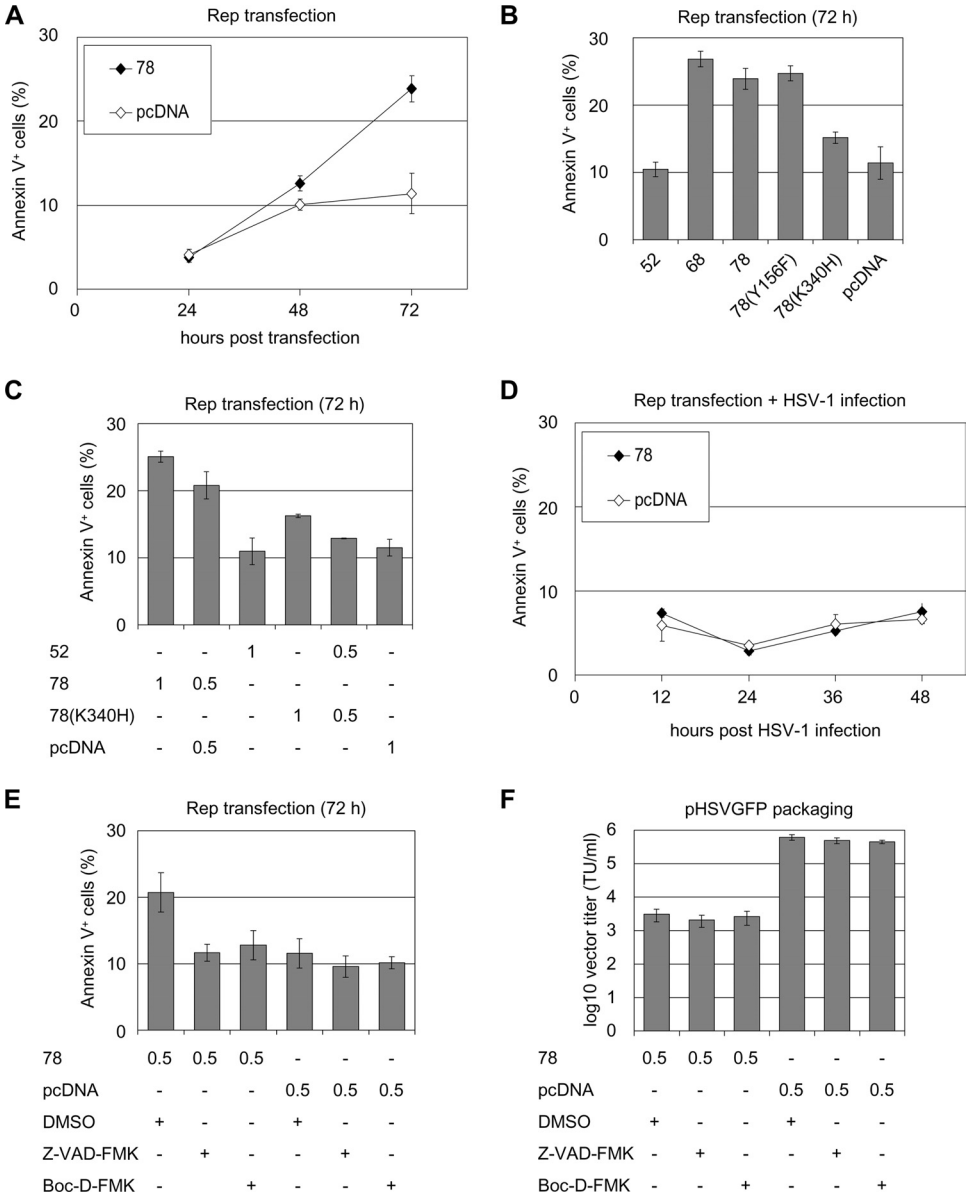


FIG. 4. Role of apoptosis in Rep-mediated inhibition of HSV-1 replication. (A) Vero cells were transfected with 0.5 μg of pcDNA.Rep78 or empty pcDNA vector together with 0.25 μg pEGFP-N3. At the indicated time points, the cells were stained with Cy5-conjugated annexin V and analyzed by flow cytometry with filters specific for EGFP (transfected cells) and Cy5 (annexin V^+ cells). (B) Vero cells were transfected with 0.5 μg of pcDNA.Rep constructs or empty pcDNA vector together with 0.25 μg pEGFP-N3 and analyzed 72 h later by annexin V staining as described for panel A. (C) Vero cells were transfected with the indicated amounts of pcDNA.Rep constructs or empty pcDNA vector (in μg) together with 0.25 μg pEGFP-N3 and analyzed 72 h later by annexin V staining as described for panel A. (D) Vero cells were transfected with 0.5 μg of pcDNA.Rep78 or empty pcDNA vector together with 0.25 μg pEGFP-N3 and subsequently infected with HSV-1 at an MOI of 10 PFU. At the indicated time points, the cells were analyzed by annexin V staining as described for panel A. (E) Vero cells were transfected with the indicated amounts of pcDNA.Rep78 or empty pcDNA vector (in μg) and incubated in the presence of the caspase inhibitor Z-VAD-FMK or Boc-D-FMK (100 μM each) or the solvent DMSO as a control. At 72 h after transfection, the cells were analyzed by annexin V staining as described for panel A. (F) Vero 2-2 cells were transfected with pHSVGFP, fHSVΔpacΔ27Δkn, pEBHICP27, and the indicated amounts of the pcDNA.Rep constructs or empty pcDNA vector (in μg). The cells were then incubated in the presence of the caspase inhibitor Z-VAD-FMK or Boc-D-FMK (100 μM each) or the solvent DMSO as a control. The pHSVGFP amplicon vectors were harvested at 72 h after transfection and titrated on Vero cells. All the data in this figure are shown as means ± SD from triplicate experiments.

Rep78, and Rep78(Y156F), while Rep52, Rep78(K340H), and the empty vector induced only low levels of apoptosis (Fig. 4B). Analogous to Rep-mediated inhibition of HSV-1 replication, Rep-mediated apoptosis required DNA-binding and ATPase/helicase activities to be on the same Rep molecule, since *trans*-complementation of DNA-binding and ATPase/helicase activities did not restore efficient induction of apoptosis (Fig. 4C). The Rep activities required for induction of apoptosis are summarized in Table 1.

The finding that the requirements for Rep-mediated apop-

tosis perfectly coincide with those for Rep-mediated inhibition of HSV-1 replication may suggest that the induction of apoptosis is part of the mechanism by which Rep inhibits HSV-1 replication. We therefore determined if apoptosis is indeed induced in Rep-expressing and HSV-1 infected cells and if pharmacological inhibition of apoptosis could prevent Rep-mediated inhibition of HSV-1 replication. To first find out if Rep induces apoptosis during HSV-1 infection, cells were transfected with either the Rep78 plasmid or the empty vector and infected immediately after transfection with HSV-1 at a high MOI (10 PFU). Cells were then harvested every 12 h until the CPE reached 100% (48 h p.i.) and analyzed by annexin V staining. As shown in Fig. 4D, annexin V staining remained low throughout the HSV-1 infection both in Rep78-expressing cells and in cells transfected with the empty vector, showing that the presence of Rep78 does not lead to apoptosis in the course of HSV-1 infection. This result is not unexpected, since HSV-1 has previously been shown to encode antiapoptotic factors (reviewed in reference 58). To assess the effect of pharmacological inhibition of caspases on Rep-mediated inhibition of HSV-1 replication, cells transfected with Rep78 or the empty vector were treated with two different broad-range caspase inhibitors, Z-VAD-FMK and Boc-D-FMK. As shown in Fig. 4E, both inhibitors prevented Rep-mediated apoptosis. However, the presence of caspase inhibitors did not prevent Rep-mediated inhibition of HSV-1 replication (Fig. 4F).

In summary, these data show that the Rep protein *per se* induces apoptosis in Vero cells and that the activities of Rep required for this effect precisely coincide with those required for inhibition of HSV-1 (i.e., DNA-binding and ATPase/helicase activities on the same Rep molecule). However, Rep does not induce apoptosis during HSV-1 infection, nor does treatment with caspase inhibitors prevent Rep-mediated inhibition of HSV-1 replication, suggesting that the execution of apoptosis is not part of the mechanism by which Rep inhibits HSV-1 replication.

The activities of Rep required for inhibition of HSV-1 replication coincide with those required for induction of DNA damage. Expression of Rep78 has previously been shown to induce DNA damage in U2OS and HeLa cells (5). The resulting DNA damage response has been shown to be mediated by ataxia telangiectasia-mutated (ATM) and to result in the activation of the checkpoint effector kinase Chk2 and histone H2AX. The authors postulated that Rep endonuclease activity induced nicks in the cellular DNA and that this was responsible for the DNA damage response. To reach that conclusion, Berthet and coworkers (5) used a mutant Rep protein lacking DNA-binding activity, Rep78Δ1–171, as well as the Rep78(K340H) mutant lacking ATPase/helicase activity (and consequently endonuclease activity on dsDNA). However, they did not use the Rep78(Y156F) mutant lacking endonuclease activity and retaining ATPase/helicase activity. Their data therefore do not elucidate if endonuclease or, rather, ATPase/helicase activity is required for the induction of cellular DNA damage.

We therefore assessed the requirements of Rep for the induction of a DNA damage response using our panel of Rep mutants. For this, Vero cells were transfected with Rep-encoding plasmids or a plasmid expressing EGFP as a negative control. Transfected cells were identified by staining with Rep-specific antibodies or by EGFP fluorescence. As markers of a DNA damage response, we used antibodies specific for hyperphosphorylated (i.e., activated) ATM (p-ATM S1981), RPA (p-RPA32 S4/S8), and H2AX (γH2AX S139). In Rep78-transfected cells, the activation of all three markers was observed as early as 24 h after transfection (data not shown). Such activation was also observed in cells treated with broad-spectrum caspase inhibitors, demonstrating that it was not a consequence of Rep-mediated apoptosis (data not shown). To identify the Rep activities required for activation of the DNA damage responses, cells were transfected with our panel of Rep plasmids and analyzed 48 h later by staining for the DNA damage markers. Examples of Rep78-transfected cells scored as positive or EGFP-transfected cells scored as negative are shown in Fig. 5A. Rep-positive cells showing an activated DNA damage response also showed a somewhat altered chromatin structure (Fig. 5A), corroborating the notion that Rep damages cellular chromatin (5). Activation of ATM, RPA, and H2AX was observed in approximately 30 to 50% of cells transfected with Rep68, Rep78, and Rep78(Y156F), while transfection with Rep52 and Rep78(K340), as well as a combination of the two, did not induce more DNA damage than the negative control (Fig. 5B).

Taken together, these data demonstrate that Rep induces a cellular DNA damage response in Vero cells characterized by hyperphosphorylation of ATM, RPA, and H2AX and that this requires DNA-binding and ATPase/helicase activities on the same Rep molecule. As such, they show that the activities of Rep required for induction of cellular DNA damage precisely coincide with those required for the inhibition of HSV-1 replication and induction of apoptosis (summarized in Table 1).

The hyperphosphorylation of RPA *per se* is not responsible for the inhibition of HSV-1 replication. It has recently become clear that differential DNA damage responses are induced during HSV-1 and AAV replication. Specifically, HSV-1 infection induces hyperphosphorylation of ATM (44, 70) and H2AX (84), but not RPA (85, 86). In contrast, productive AAV infection leads to hyperphosphorylation of ATM, RPA, and H2AX (68). We therefore hypothesized that components of the DNA damage response elicited by Rep, specifically the hyperphosphorylation of RPA, may be involved in the inhibition of HSV-1 replication. HSV-1 replication does not induce hyperphosphorylation of RPA unless the HSV-1 DNA polymerase is blocked by inhibitors such as PAA or acyclovir (85, 86). In addition, endogenous p-RPA is sequestered into virus-induced chaperone-enriched (VICE) domains during productive replication (84), suggesting that p-RPA may be inhibitory to HSV-1 replication and that HSV-1 may prevent such inhibition by excluding p-RPA from HSV-1 RCs. To assess if hyperphosphorylation of RPA is mediating the inhibition of HSV-1 replication, we induced hyperphosphorylation of RPA by pretreatment of cells with hydroxyurea (HU) (47) or camptothecin (CPT) (69) or infection with UV-inactivated AAV (UV-AAV) (26, 40) and assessed the effect of these treatments on HSV-1 replication. While HU and CPT treatments result in damaging of cellular DNA, infection of cells with UV-AAV induces a DNA damage response without actually damaging cellular DNA (26, 40). The DNA damage response to UV-AAV resembles stalled replication fork signaling and involves DNA polymerase δ, ATR, TopBP1, RPA, the Rad9/Rad1/

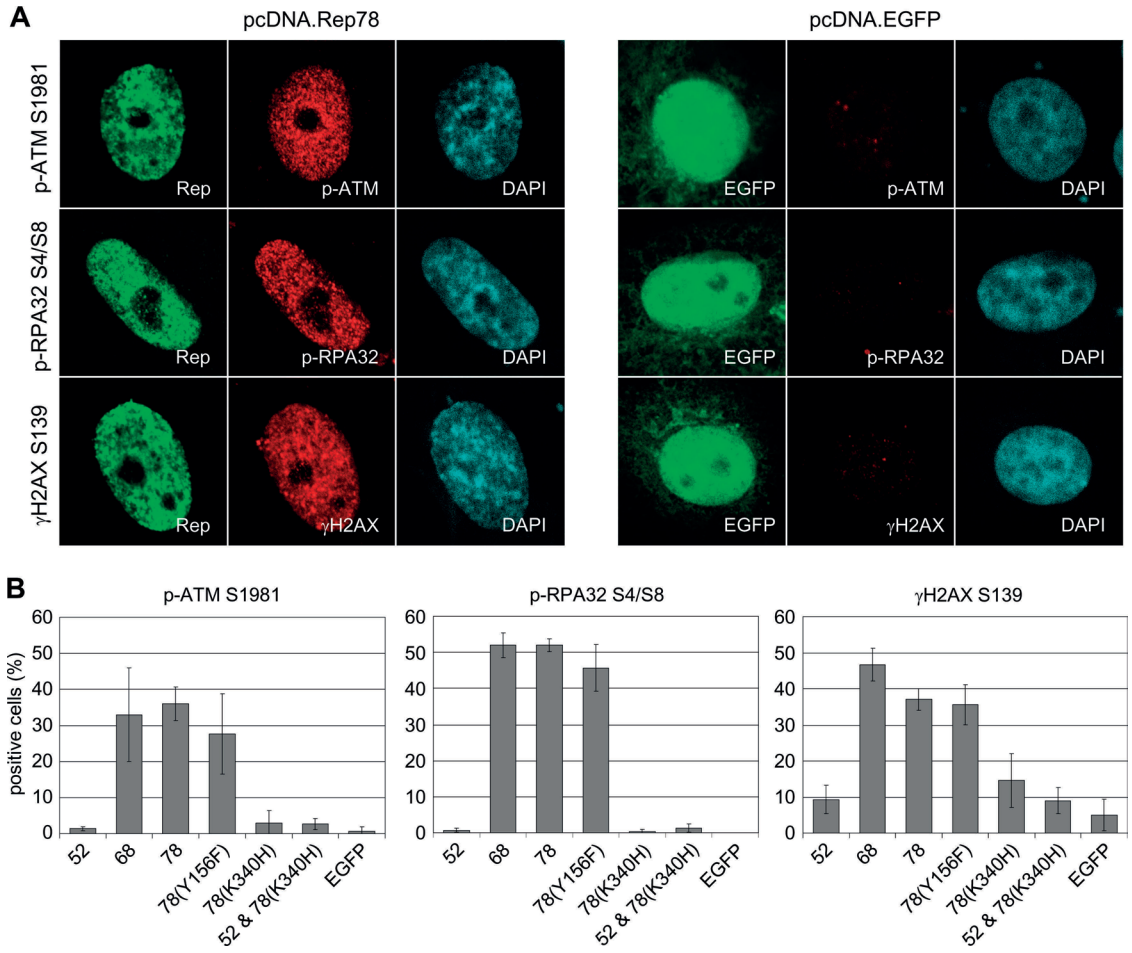


FIG. 5. Screening of Rep proteins for induction of cellular DNA damage. (A) Examples of Rep78-transfected cells scored as positive and EGFP-transfected cells scored as negative for DNA damage markers. Vero cells were transfected with 0.5 μg of pcDNA.Rep78 or pcDNA.EGFP. At 48 h after transfection, the cells were fixed and stained with antibodies specific for p-ATM S1981, p-RPA32 S4/S8, or γH2AX S139 (red). pcDNA.Rep78-transfected cells were identified by staining with Rep-specific antibodies, while pcDNA.EGFP-transfected cells were identified by EGFP fluorescence (green). Cellular chromatin was stained with DAPI (cyan). Images were recorded by CLSM and show single *z* stacks of representative cells. (B) Quantification of cells staining positive for DNA damage markers. Vero cells were transfected with 0.5 μg of the indicated pcDNA.Rep constructs or pcDNA.EGFP and stained as described for panel A. Rep^+ or $EGFP^+$ cells ($n = 100$) were scored for staining of p-ATM S1981, p-RPA32 S4/S8, or γH2AX S139. The data are shown as means ± SD from three independent experiments.

Hus1 complex, H2AX, and Chk1 but not ATM or NBS1 (26, 40). Although the cellular DNA itself is not damaged, such DNA damage signaling has profound effects on the cell cycle in that it results in G_2 arrest (40, 61).

We first confirmed the induction of a DNA damage response in Vero cells upon treatment with HU (2.5 mM), CPT (1 μM), and UV-AAV (MOI of 10^4 and 5×10^5 gcp). All three treatments induced a strong hyperphosphorylation of RPA (p-RPA32 S4/S8) and H2AX (γH2AX S139) compared to that in control cells (Fig. 6A to C). ATM was strongly hyperphosphorylated (p-ATM S1981) upon HU and CPT treatment but only weakly hyperphosphorylated upon infection with UV-AAV (Fig. 6A to C), suggesting that HU and CPT, but not UV-AAV, induced significant double-strand breaks. We next confirmed efficient inactivation of AAV by assessing the levels of Rep proteins by Western blotting. While Rep proteins were readily expressed in AAV/HSV-1-coinfected Vero cells, no Rep-specific bands were observed in UV-AAV/HSV-1-coinfected cells, confirming that UV inactivation was sufficient (Fig. 6D). Finally, Vero cells were treated with HU, CPT, and UV-AAV for the indicated times, washed thoroughly, and then infected with a recombinant HSV-1 expressing EGFP (HSV-1 C12) at high a MOI (5 PFU). When CPE reached 100%, the HSV-1 C12 produced was harvested and titrated. As shown in Fig. 6E, HU treatment significantly inhibited HSV-1 replication compared to untreated cells, while both UV-AAV and CPT had no significant effect on HSV-1 productivity.

Taken together, these results demonstrate that although all three pretreatments, HU, CPT, and UV-AAV, strongly induced the hyperphosphorylation of RPA, only HU led to significant inhibition of HSV-1 replication, while CPT and UV-AAV did not affect HSV-1 productivity. As such, they suggest that the hyperphosphorylation of RPA *per se* is not inhibitory for HSV-1 replication and that HU inhibits HSV-1 replication

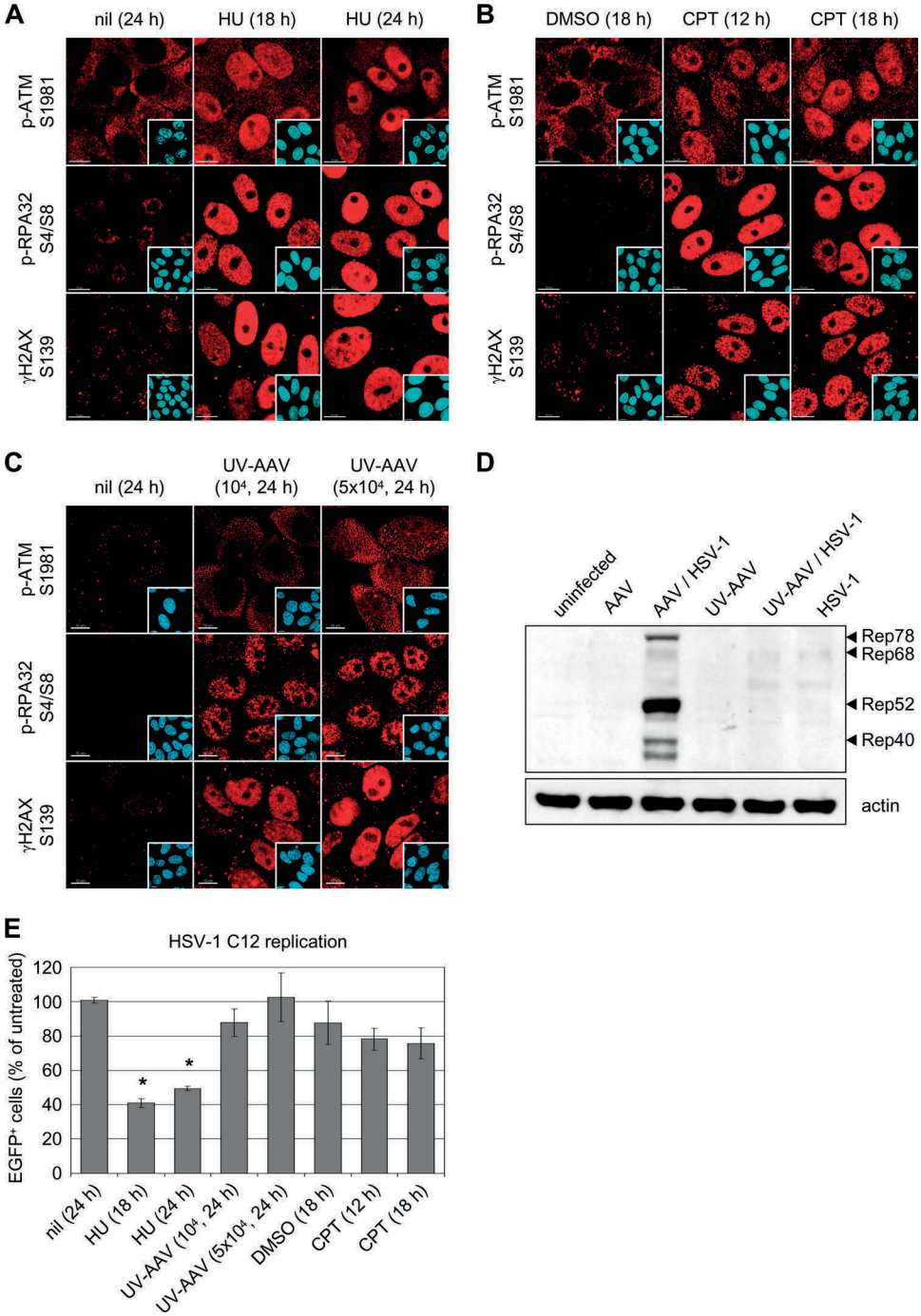


FIG. 6. (A to C) Immunofluorescence staining for DNA damage markers upon HU, CPT, and UV-AAV treatment. Vero cells were treated for the indicated times with HU (2.5 mM) or left untreated (nil) (A), with CPT (1 μM) or its solvent DMSO alone (B), or with UV-AAV (MOI of 10^4 or 5×10^4 gcp) or left untreated (nil) (C). The cells were then fixed and stained for p-ATM S1981, p-RPA32 S4/S8, or γH2AX S139 (red). Chromatin was stained with DAPI (blue; inset). Scale bars, 10 μm. Images were recorded by CLSM and show single *z* stacks of representative cells. (D) Western blot for Rep proteins upon live AAV or UV-AAV infection. Vero cells were left uninfected or infected with live AAV or UV-AAV (MOI of 5×10^4 gcp). Twenty-four hours later, the cells were superinfected with HSV-1 (MOI of 5 PFU) or left uninfected. Twenty-four hours after HSV-1 infection, the cells were lysed and processed for Western blotting with a Rep-specific MAb. Detection of actin served as a loading control. (E) HSV-1 replication in cells pretreated with HU, CPT, and UV-AAV. Vero cells were left untreated (nil) or treated with HU (2.5 mM), UV-AAV (MOI of 10^4 or 5×10^4 gcp), and CPT (1 μM) or its solvent DMSO alone for the indicated times. After three washes in PBS, the cells were infected with HSV-1 C12 (expressing EGFP) at an MOI of 5 PFU. At 24 h after HSV-1 C12 infection, virus was harvested and titrated on Vero cells by enumerating $EGFP^+$ cells. The data are shown as means ± SD from triplicate experiments. The asterisks indicate statistically significant reductions compared to untreated cells ($P < 0.05$).

by a different mechanism, presumably the depletion of deoxynucleoside triphosphate (dNTP) pools by inhibition of ribonucleotide reductase (2).

DISCUSSION

In a first set of experiments, we characterized the inhibitory effect of Rep on HSV-1 replication. For this purpose, we assessed the levels of infectious HSV-1 particles, the levels of replicated HSV-1 DNA, and the levels of expressed HSV-1 gene products in the presence of Rep78 expressed from the HCMV IE1 enhancer/promoter. The presence of Rep78 resulted in a dose-dependent inhibition of HSV-1 productivity, DNA replication, and gene expression. The inhibitory effect on the latter was not uniform in that Rep78 had a stronger effect on the late gene products VP16, VP22, and gC than on the IE gene product ICP4 and the early gene products UL9, ICP8, UL5, and UL42 (Fig. 2). These findings are consistent with our previous observations that in the situation of AAV/HSV-1 coinfection, HSV-1 replication is inhibited mainly at the levels of DNA replication and late gene expression, while IE and early gene expression is only marginally reduced (31). It appeared, however, that the expression of Rep78 from the HCMV IE1 enhancer/promoter had a more pronounced effect on the levels of HSV-1 IE and early gene products than AAV coinfection, an observation which may be explained by the different expression kinetics of *rep*. Specifically, high-level expression of the AAV *rep* gene from its native promoters (p5 and p19) requires transactivation by the HSV-1 IE early gene products ICP0, ICP4, and ICP22 (3). Consequently, in the situation of AAV/HSV-1 coinfection, expression of the *rep* gene is expected to occur with kinetics similar to that of HSV-1 early genes. In the present study, in contrast, Rep78 was expressed with IE kinetics, which may explain why the inhibitory effect on HSV-1 IE and early gene products was more pronounced than in the situation of AAV/HSV-1 coinfection (31). However, the reduced levels of HSV-1 early gene products, in particular the HSV-1 replication factors, appeared not to be responsible for the inhibition of HSV-1 DNA replication, since Rep inhibited HSV-1 DNA replication even in the presence of overexpressed HSV-1 replication factors (Fig. 3). In addition, the Rep levels employed were compatible with AAV DNA replication, which also depends on the HSV-1 replication machinery (Fig. 2). These findings suggest that Rep may have a direct inhibitory effect on HSV-1 DNA replication.

In a second set of experiments, we identified the Rep activities required for inhibition of HSV-1 replication. The results demonstrate that the inhibition of HSV-1 replication requires Rep DNA-binding and ATPase/helicase activities on the same Rep molecule, while the C terminus of Rep78 and Rep endonuclease activity are not required (Fig. 2 and Table 1). The finding that Rep DNA-binding and ATPase/helicase activities are required and that they need to be present on the same Rep molecule is consistent with a model in which the inhibition of HSV-1 replication involves the interaction of Rep with DNA and in which the Rep helicase activity needs to be targeted to a dsDNA substrate via the DNA-binding domain. Theoretically, the inhibition of HSV-1 replication could result either from a direct effect of Rep on HSV-1 DNA or else from an effect on cellular DNA. Rep is known to have a variety of effects on the host cell, including the induction of DNA damage, cell cycle arrest, and apoptosis (5, 65, 66, 68). Apoptosis has been shown to require Rep DNA-binding and ATPase/helicase activities (66), notably the same activities required for inhibition of HSV-1 replication. We therefore hypothesized that Rep-mediated induction of apoptosis may be part of the mechanism by which Rep inhibits HSV-1 replication. Our data show that although the activities required for induction of apoptosis and for inhibition of HSV-1 replication coincide precisely (Fig. 4 and Table 1), execution of apoptosis is not part of the inhibitory mechanism. First, apoptosis did not occur in cells expressing Rep78 and infected with HSV-1, and second, treatment with caspase inhibitors did not abolish the inhibitory activity of Rep on HSV-1 replication (Fig. 4). We concluded from these data that the ability of Rep to induce apoptosis must be closely linked to its ability to inhibit HSV-1 replication, possibly by acting in the same pathway. Furthermore, the finding that the execution of apoptosis is not required for inhibition of HSV-1 replication suggests that Rep-mediated apoptosis may only be the consequence of an upstream Rep effect leading to inhibition of HSV-1 replication. A possible candidate for such an upstream effect is the ability of Rep to induce cellular DNA damage, an effect which results in DNA damage signaling, cell cycle arrest, and presumably apoptosis (5). We therefore assessed which activities of Rep were required for the induction of DNA damage. Intriguingly, the Rep activities required for induction of cellular DNA damage precisely corresponded to those required for induction of apoptosis and inhibition of HSV-1 replication, corroborating the notion that these three Rep effects are linked in a common pathway (Fig. 5 and Table 1). Consistent with previous findings, expression of Rep78 resulted in the hyperphosphorylation of ATM (p-ATM S1981) and H2AX (γH2AX S139) (5, 68). The phosphatidylinositol 3-kinase-like kinase (PIKK) ATM is a master regulator of the cellular response to double-strand breaks and activates a variety of substrates involved in cell cycle checkpoints and DNA repair. ATM protein kinase activity is activated upon DNA damage stimuli, and this activation has been attributed to intermolecular autophosphorylation on serine 1981 and dissociation of multimeric ATM to an active, monomeric form (reviewed in reference 42). ATM has been shown to be essential for Rep78-induced DNA damage signaling, in that expression of Rep78 did not result in cell cycle arrest and activation of H2AX in ATM-null cell lines (5). H2AX is a histone protein which can be hyperphosphorylated at serine 139 by ATM in response to double-strand breaks or, alternatively, by ATM- and Rad3-related (ATR) kinase in response to replication stalling and single-strand breaks and is involved in the assembly and retention of DNA repair factors at the DNA lesion (reviewed in reference 24). We also observed the hyperphosphorylation of RPA (p-RPA32 S4/S8), a heterotrimeric ssDNA-binding protein consisting of 70-, 32-, and 14-kDa subunits which is involved in diverse processes such as DNA replication, DNA repair, recombination, and DNA damage checkpoints (reviewed in reference 92). RPA hyperphosphorylation occurs upon DNA damage signaling and can be mediated by the PIKKs ATM, ATR, and DNA-dependent protein kinase (DNA-PK), depending on the nature of the DNA lesion (reviewed in references 6 and 19). Theoretically, there are two

different possible explanations for the observation that the ability of Rep to induce DNA damage correlates with its ability to inhibit HSV-1 replication. First, the Rep-induced DNA damage itself, presumably acting on both cellular and HSV-1 DNAs, could be responsible for inhibition of HSV-1 replication. Second, the cellular response to Rep-induced DNA damage could be responsible for inhibition of HSV-1 replication by creating a cellular environment unfavorable for HSV-1 replication. Indeed, AAV and HSV-1 have recently been demonstrated to induce differential DNA damage signaling and to be differentially affected by DNA damage and repair proteins. Specifically, productive AAV infection induces the hyperphosphorylation of ATM, RPA, and H2AX (68), while HSV-1 infection leads to hyperphosphorylation of ATM (44, 70) and H2AX (84) but not RPA (85, 86). To find out if the Rep-mediated activation of RPA may be responsible for inhibition of HSV-1 replication, we assessed the replication efficiency of HSV-1 in cells in which RPA hyperphosphorylation had previously been induced by treatment with HU or CPT or by infection with UV-AAV. Although all three agents efficiently induced hyperphosphorylation of RPA, only HU pretreatment significantly inhibited HSV-1 replication (Fig. 6). This suggests that Rep-induced activation of RPA *per se* is not responsible for the inhibition of HSV-1 replication and that the observed inhibition of HSV-1 replication by HU involves mechanisms other than RPA hyperphosphorylation, presumably the depletion of dNTP pools by inhibition of ribonucleotide reductase (2). These findings may be interpreted in at least two different ways. First, they may mean that the hyperphosphorylation of RPA does not interfere with efficient HSV-1 replication. Second, they may mean that HSV-1 can efficiently circumvent a potential inhibitory effect by sequestering p-RPA away from its RCs into VICE domains (84).

Although the finding that inhibition of HSV-1 replication requires Rep DNA-binding and ATPase/helicase activities on the same Rep molecule is consistent with the idea that the inhibition of HSV-1 replication involves the interaction of Rep with target DNA, it is also conceivable that Rep inhibits HSV-1 replication by its interaction with the HSV-1 DNA replication complex. It is thought that the direct interaction between Rep and ICP8 (33, 75) leads to the recruitment of the HSV-1 DNA replication complex to AAV replication origins. Consistent with this, essential roles of ICP8, the HSV-1 helicase-primase complex, and the HSV-1 DNA polymerase holoenzyme in AAV replication have been demonstrated (3, 82). However, it is also possible that the Rep-ICP8 interaction directs Rep to DNA replication complexes on HSV-1 replication origins, where it possibly interferes with efficient HSV-1 DNA replication. In analogy, the interaction of Rep with RPA (75) might direct Rep to cellular DNA replication complexes, leading to inhibition of cellular DNA replication. Direct interactions with the ssDNA-binding proteins ICP8, RPA, and Ad-DBP have been demonstrated for Rep68 and Rep78 (33, 75), and these interactions have been shown to enhance binding of Rep68 and Rep78 to the Rep-binding site (RBS) (75). The enhancement of Rep DNA binding by all three ssDNA-binding proteins was also observed for a mutant Rep protein lacking ATPase/helicase activity, Rep68(K340H/Y121H), implying that the mutant Rep protein can still interact with ICP8, RPA, and Ad-DBP (75). As such, these findings are consistent with the idea that the inability of the Rep78(K340H) mutant to inhibit the replication of HSV-1 is not due to a loss of the direct interaction with ICP8 but rather is due to the loss of the ATPase/helicase activity itself. We therefore consider it more likely that the inhibition of HSV-1 replication involves the interaction of Rep with target DNA rather than that with ICP8.

How could Rep DNA-binding and ATPase/helicase activities result in cellular DNA damage and apoptosis, as well as inhibition of HSV-1 replication? Rep displays sequence-specific binding to dsDNA, which targets the protein to the RBSs within the AAV replication origins, as well as to its preintegration site on human chromosome 19 (*AAVS1*). The sequence-specific DNA-binding activity requires the amino-terminal 224 amino acids, which are present in Rep68 and Rep78 but not in Rep40 and Rep52 (60). The sequence recognized by Rep consists of an array of GAGC tetranucleotide repeats, with the binding affinity being determined by the number of perfect GAGC repeats present as well as secondary structures neighboring the RBSs (14, 15, 30, 51, 52, 62, 87). The AAV genome, for instance, contains RBSs within the ITR replication origins (GAGC GAGC GAGC GCGC), as well as within the p5 promoter (GAGT GAGC ACGC AGGG), with the binding affinity of Rep being higher for the ITR than for the p5 RBS (30). In addition to the RBS present in the *AAVS1* preintegration site (CAGC GAGC GAGC GAGC), the human genome contains 2×10^5 potential RBSs if they are defined as GAGY GAGC motifs (90). By applying the same definition, nine minimal RBSs can be found in the HSV-1 genome. However, the sequence requirements for low-affinity Rep binding are less stringent, in that a single GAGC repeat followed by a run of G bases is sufficient (15), suggesting that the above numbers of potential RBSs within the cellular and HSV-1 genomes are underestimated. In contrast, appropriately spaced terminal resolution sites (TRSs), where Rep endonuclease activity can induce a site- and strand-specific nick, are present only in the AAV replication origins and in *AAVS1*, and therefore, only these sites function as Rep-dependent replication origins. The fact that the cellular RBSs outside *AAVS1* lack functional TRSs is thought to determine the specificity of AAV integration into *AAVS1* (45, 87, 90). In the absence of a helper virus, Rep concentrations are low and Rep is expected to be targeted to the rather strong RBSs within the AAV replication origins and the *AAVS1* preintegration site, resulting in site-specific integration and establishment of latent infection. In the presence of a helper virus such as HSV-1, Rep concentrations are high and Rep is expected to additionally bind to more degenerate RBSs present on the cellular and HSV-1 genomes. Binding of Rep to degenerate RBSs lacking an appropriately spaced TRS is expected to be followed by unwinding via the helicase activity but not by nicking. The helicase activity of Rep has no specific sequence requirement for unwinding of dsDNA substrates. However, Rep helicase activity requires prior binding to the DNA substrate. If the substrate is blunt-ended dsDNA, Rep68/78 helicase activity requires the presence of an RBS, to which the protein binds via its N-terminal DNA-binding domain (88, 91). If the DNA substrate does not contain an RBS, Rep68/78 and Rep52 helicase activity require a single-stranded 3′ tail, to which Rep binds and along which it moves in a 3′-to-5′ direction while unwinding the stretch of

dsDNA (74, 88, 91). In contrast, Rep40 not only can bind to 3′ single-stranded tails but in addition can initiate helicase activity on a blunt-ended dsDNA substrate (18). Our finding that Rep68 and Rep78, but not Rep52, inhibit HSV-1 replication suggests that the substrate for Rep helicase activity is dsDNA and that Rep binds to its substrate via its sequence-specific DNA-binding domain. It is conceivable that Rep DNA binding followed by unwinding of the DNA double strand interferes with transcription and replication, since it may block access of transcription and replication factors. However, the mechanism by which Rep DNA binding and unwinding induces a cellular DNA damage response is more difficult to understand. One straightforward explanation would be that the ssDNA exposed upon Rep helicase activity is sensed as DNA damage, resulting in the activation of the corresponding DNA damage signaling. Accumulation of ssDNA as it occurs upon replication stalling leads to activation of the ATR/ATRIP signaling cascade, resulting in activation of Chk1 and ultimately G_2 arrest (reviewed in reference 19). However, Rep does not induce activation of ATR and Chk1 but rather that of ATM, Chk2, H2AX, and RPA (5) (Fig. 5). Since activation of ATM and its downstream target Chk2 is generally thought to mark the occurrence of double-strand breaks, it is currently not clear how exactly Rep induces DNA damage and how the corresponding damage signaling is activated. It was previously suggested that Rep damages cellular DNA by inducing nicks via its endonuclease activity (5). Our finding that the endonuclease activity of Rep is not required for induction of cellular DNA damage (Fig. 5), however, suggests that the mechanism is more complex. It is possible that the process of Rep DNA binding and unwinding recruits other cellular factors which then induce the actual DNA damage. Consistent with such a hypothesis, Rep has been shown to interact with a variety of cellular proteins involved in DNA replication and repair (57).

Whatever the mechanism, our data show that Rep DNA-binding and ATPase/helicase activities on the same Rep molecule are required for induction of DNA damage and apoptosis, as well as for inhibition of HSV-1 replication, and as such they suggest that these three processes are closely linked. Since execution of apoptosis is not involved in inhibition of HSV-1 replication (Fig. 4), we consider it possible that the ability of Rep to induce DNA damage is actually responsible for inhibition of HSV-1. Such Rep-induced DNA damage might occur not only on cellular DNA but also directly on HSV-1 DNA, the latter of which would presumably interfere with HSV-1 transcription and DNA replication. On AAV DNA, in contrast, the process of Rep binding and unwinding is followed by nicking and is targeted to the AAV replication origins (ITR and p5) where it fulfills an essential step in the AAV DNA replication cycle, i.e., the creation of a free 3′ end for repair synthesis of the ITRs. Finally, the inhibition of HSV-1 replication would confer a competitive advantage to AAV, since it would limit the competition of HSV-1 for replication factors. Although such an inhibitory mechanism would not be specific for HSV-1 DNA replication in that it is also expected to affect HSV-1 transcription, it would still allow the largely undisturbed expression of the HSV-1 helper factors for AAV replication, since *rep* expression occurs with early kinetics and therefore is expected to have only a limited inhibitory effect on HSV-1 IE and early gene transcription.

At present, such a model certainly remains speculative, since several aspects still need to be addressed experimentally, in particular the interaction of Rep with cellular and HSV-1 DNAs and the consequences of this interaction for the transcription and replication of these DNA substrates. Nonetheless, it represents an intriguing explanation for the observation that AAV Rep can inhibit the replication of such diverse DNA substrates as cellular (5, 65, 89), Ad (77), simian virus 40 (SV40) (89), and HSV-1 (32) DNAs, inasmuch as it would predict inhibited replication of every DNA substrate on which Rep binding and unwinding occur at random locations and for which it is not part of the replicative cycle.

ACKNOWLEDGMENTS

We thank C. Berthet, P. Beard, G. H. Cohen, R. J. Eisenberg, S. Efstathiou, G. Elliott, R. D. Everett, R. Heilbronn, D. M. Knipe, A. Minson, H. Browne, N. D. Stow, and J. P. Trempe for providing reagents, as well as D. Winder and I. Brierley for helpful discussions and I. Heid for technical assistance.

This work was supported by grants from the Novartis Foundation for Biomedical Research (06C77 to C.F.), the Swiss National Science Foundation (3100AO-112462 to C.F.), and the National Institutes of Health (CA69246 to C.F.). D.L.G. is currently supported by a fellowship of the Swiss National Science Foundation (PBZHP3-122925). A.S. is supported by grants from the French Muscular Dystrophy Association, INSERM, and Direction Générale à la Santé.

REFERENCES

1. **Aboody-Guterman, K. S., P. A. Pechan, N. G. Rainov, M. Sena-Esteves, A. Jacobs, E. Y. Snyder, P. Wild, E. Schraner, K. Tobler, X. O. Breakefield, and C. Fraefel.** 1997. Green fluorescent protein as a reporter for retrovirus and helper virus-free HSV-1 amplicon vector-mediated gene transfer into neural cells in culture and in vivo. Neuroreport **8:**3801–3808.
2. **Adams, R. L., and J. G. Lindsay.** 1967. Hydroxyurea reversal of inhibition and use as a cell-synchronizing agent. J. Biol. Chem. **242:**1314–1317.
3. **Alazard-Dany, N., A. Nicolas, A. Ploquin, R. Strasser, A. Greco, A. L. Epstein, C. Fraefel, and A. Salvetti.** 2009. Definition of herpes simplex virus type 1 helper activities for adeno-associated virus early replication events. PLoS Pathog. **5:**e1000340.
4. **Bantel-Schaal, U., and H. zur Hausen.** 1988. Adeno-associated viruses inhibit SV40 DNA amplification and replication of herpes simplex virus in SV40-transformed hamster cells. Virology **164:**64–74.
5. **Berthet, C., K. Raj, P. Saudan, and P. Beard.** 2005. How adeno-associated virus Rep78 protein arrests cells completely in S phase. Proc. Natl. Acad. Sci. U. S. A. **102:**13634–13639.
6. **Binz, S. K., A. M. Sheehan, and M. S. Wold.** 2004. Replication protein A phosphorylation and the cellular response to DNA damage. DNA Repair **3:**1015–1024.
7. **Brister, J. R., and N. Muzyczka.** 2000. Mechanism of Rep-mediated adeno-associated virus origin nicking. J. Virol. **74:**7762–7771.
8. **Brister, J. R., and N. Muzyczka.** 1999. Rep-mediated nicking of the adeno-associated virus origin requires two biochemical activities, DNA helicase activity and transesterification. J. Virol. **73:**9325–9336.
9. **Buller, R. M., J. E. Janik, E. D. Sebring, and J. A. Rose.** 1981. Herpes simplex virus types 1 and 2 completely help adenovirus-associated virus replication. J. Virol. **40:**241–247.
10. **Carter, B. J., C. A. Laughlin, L. M. de la Maza, and M. Myers.** 1979. Adeno-associated virus autointerference. Virology **92:**449–462.
11. **Casto, B. C., J. A. Armstrong, R. W. Atchison, and W. M. Hammon.** 1967. Studies on the relationship between adeno-associated virus type 1 (AAV-1) and adenoviruses. II. Inhibition of adenovirus plaques by AAV; its nature and specificity. Virology **33:**452–458.
12. **Casto, B. C., R. W. Atchison, and W. M. Hammon.** 1967. Studies on the relationship between adeno-associated virus type I (AAV-1) and adenoviruses. I. Replication of AAV-1 in certain cell cultures and its effect on helper adenovirus. Virology **32:**52–59.
13. **Chejanovsky, N., and B. J. Carter.** 1990. Mutation of a consensus purine nucleotide binding site in the adeno-associated virus rep gene generates a dominant negative phenotype for DNA replication. J. Virol. **64:**1764–1770.
14. **Chiorini, J. A., S. M. Wiener, R. A. Owens, S. R. Kyostio, R. M. Kotin, and B. Safer.** 1994. Sequence requirements for stable binding and function of Rep68 on the adeno-associated virus type 2 inverted terminal repeats. J. Virol. **68:**7448–7457.
15. **Chiorini, J. A., L. Yang, B. Safer, and R. M. Kotin.** 1995. Determination of

adeno-associated virus Rep68 and Rep78 binding sites by random sequence oligonucleotide selection. J. Virol. **69:**7334–7338.
16. **Chiorini, J. A., B. Zimmermann, L. Yang, R. H. Smith, A. Ahearn, F. Herberg, and R. M. Kotin.** 1998. Inhibition of PrKX, a novel protein kinase, and the cyclic AMP-dependent protein kinase PKA by the regulatory proteins of adeno-associated virus type 2. Mol. Cell. Biol. **18:**5921–5929.
17. **Cohen, G. H., D. Long, and R. J. Eisenberg.** 1980. Synthesis and processing of glycoproteins gD and gC of herpes simplex virus type 1. J. Virol. **36:**429–439.
18. **Collaco, R. F., V. Kalman-Maltese, A. D. Smith, J. D. Dignam, and J. P. Trempe.** 2003. A biochemical characterization of the adeno-associated virus Rep40 helicase. J. Biol. Chem. **278:**34011–34017.
19. **Cortez, D.** 2005. Unwind and slow down: checkpoint activation by helicase and polymerase uncoupling. Genes Dev. **19:**1007–1012.
20. **Davis, M. D., J. Wu, and R. A. Owens.** 2000. Mutational analysis of adeno-associated virus type 2 Rep68 protein endonuclease activity on partially single-stranded substrates. J. Virol. **74:**2936–2942.
21. **de Oliveira, A. P., D. L. Glauser, A. S. Laimbacher, R. Strasser, E. M. Schraner, P. Wild, U. Ziegler, X. O. Breakefield, M. Ackermann, and C. Fraefel.** 2008. Live visualization of herpes simplex virus type 1 compartment dynamics. J. Virol. **82:**4974–4990.
22. **Di Pasquale, G., and J. A. Chiorini.** 2003. PKA/PrKX activity is a modulator of AAV/adenovirus interaction. EMBO J. **22:**1716–1724.
23. **Di Pasquale, G., and S. N. Stacey.** 1998. Adeno-associated virus Rep78 protein interacts with protein kinase A and its homolog PRKX and inhibits CREB-dependent transcriptional activation. J. Virol. **72:**7916–7925.
24. **Fernandez-Capetillo, O., A. Lee, M. Nussenzweig, and A. Nussenzweig.** 2004. H2AX: the histone guardian of the genome. DNA Repair **3:**959–967.
25. **Fraefel, C., A. G. Bittermann, H. Bueler, I. Heid, T. Bachi, and M. Ackermann.** 2004. Spatial and temporal organization of adeno-associated virus DNA replication in live cells. J. Virol. **78:**389–398.
26. **Fragkos, M., M. Breuleux, N. Clement, and P. Beard.** 2008. Recombinant adeno-associated viral vectors are deficient in provoking a DNA damage response. J. Virol. **82:**7379–7387.
27. **Francois, A., M. Guilbaud, R. Awedikian, G. Chadeuf, P. Moullier, and A. Salvetti.** 2005. The cellular TATA binding protein is required for rep-dependent replication of a minimal adeno-associated virus type 2 p5 element. J. Virol. **79:**11082–11094.
28. **Geoffroy, M. C., A. L. Epstein, E. Toublanc, P. Moullier, and A. Salvetti.** 2004. Herpes simplex virus type 1 ICP0 protein mediates activation of adeno-associated virus type 2 rep gene expression from a latent integrated form. J. Virol. **78:**10977–10986.
29. **Glauser, D. L., M. Ackermann, O. Saydam, and C. Fraefel.** 2006. Chimeric herpes simplex virus/adeno-associated virus amplicon vectors. Curr. Gene Ther. **6:**315–324.
30. **Glauser, D. L., O. Saydam, N. A. Balsiger, I. Heid, R. M. Linden, M. Ackermann, and C. Fraefel.** 2005. Four-dimensional visualization of the simultaneous activity of alternative adeno-associated virus replication origins. J. Virol. **79:**12218–12230.
31. **Glauser, D. L., R. Strasser, A. S. Laimbacher, O. Saydam, N. Clément, R. M. Linden, M. Ackermann, and C. Fraefel.** 2007. Live covisualization of competing adeno-associated virus and herpes simplex virus type 1 DNA replication: molecular mechanisms of interaction. J. Virol. **81:**4732–4743.
32. **Heilbronn, R., A. Burkle, S. Stephan, and H. zur Hausen.** 1990. The adeno-associated virus rep gene suppresses herpes simplex virus-induced DNA amplification. J. Virol. **64:**3012–3018.
33. **Heilbronn, R., M. Engstler, S. Weger, A. Krahn, C. Schetter, and M. Boshart.** 2003. ssDNA-dependent colocalization of adeno-associated virus Rep and herpes simplex virus ICP8 in nuclear replication domains. Nucleic Acids Res. **31:**6206–6213.
34. **Heilbronn, R., and H. zur Hausen.** 1989. A subset of herpes simplex virus replication genes induces DNA amplification within the host cell genome. J. Virol. **63:**3683–3692.
35. **Heister, T., I. Heid, M. Ackermann, and C. Fraefel.** 2002. Herpes simplex virus type 1/adeno-associated virus hybrid vectors mediate site-specific integration at the adeno-associated virus preintegration site, AAVS1, on human chromosome 19. J. Virol. **76:**7163–7173.
36. **Hirt, B.** 1969. Replicating molecules of polyoma virus DNA. J. Mol. Biol. **40:**141–144.
37. **Horer, M., S. Weger, K. Butz, F. Hoppe-Seyler, C. Geisen, and J. A. Kleinschmidt.** 1995. Mutational analysis of adeno-associated virus Rep protein-mediated inhibition of heterologous and homologous promoters. J. Virol. **69:**5485–5496.
38. **Im, D. S., and N. Muzyczka.** 1990. The AAV origin binding protein Rep68 is an ATP-dependent site-specific endonuclease with DNA helicase activity. Cell **61:**447–457.
39. **Im, D. S., and N. Muzyczka.** 1989. Factors that bind to adeno-associated virus terminal repeats. J. Virol. **63:**3095–3104.
40. **Jurvansuu, J., K. Raj, A. Stasiak, and P. Beard.** 2005. Viral transport of DNA damage that mimics a stalled replication fork. J. Virol. **79:**569–580.
41. **Knipe, D. M., D. Senechek, S. A. Rice, and J. L. Smith.** 1987. Stages in the nuclear association of the herpes simplex virus transcriptional activator protein ICP4. J. Virol. **61:**276–284.
42. **Kurz, E. U., and S. P. Lees-Miller.** 2004. DNA damage-induced activation of ATM and ATM-dependent signaling pathways. DNA Repair **3:**889–900.
43. **Kyostio, S. R., and R. A. Owens.** 1996. Identification of mutant adeno-associated virus Rep proteins which are dominant-negative for DNA helicase activity. Biochem. Biophys. Res. Commun. **220:**294–299.
44. **Lilley, C. E., C. T. Carson, A. R. Muotri, F. H. Gage, and M. D. Weitzman.** 2005. DNA repair proteins affect the lifecycle of herpes simplex virus 1. Proc. Natl. Acad. Sci. U. S. A. **102:**5844–5849.
45. **Linden, R. M., E. Winocour, and K. I. Berns.** 1996. The recombination signals for adeno-associated virus site-specific integration. Proc. Natl. Acad. Sci. U. S. A. **93:**7966–7972.
46. **Lux, K., N. Goerlitz, S. Schlemminger, L. Perabo, D. Goldnau, J. Endell, K. Leike, D. M. Kofler, S. Finke, M. Hallek, and H. Buning.** 2005. Green fluorescent protein-tagged adeno-associated virus particles allow the study of cytosolic and nuclear trafficking. J. Virol. **79:**11776–11787.
47. **Manthey, K. C., S. Opiyo, J. G. Glanzer, D. Dimitrova, J. Elliott, and G. G. Oakley.** 2007. NBS1 mediates ATR-dependent RPA hyperphosphorylation following replication-fork stall and collapse. J. Cell Sci. **120:**4221–4229.
48. **Marsden, H. S., A. M. Cross, G. J. Francis, A. H. Patel, K. MacEachran, M. Murphy, G. McVey, D. Haydon, A. Abbotts, and N. D. Stow.** 1996. The herpes simplex virus type 1 UL8 protein influences the intracellular localization of the UL52 but not the ICP8 or POL replication proteins in virus-infected cells. J. Gen. Virol. **77:**2241–2249.
49. **Marsden, H. S., G. W. McLean, E. C. Barnard, G. J. Francis, K. MacEachran, M. Murphy, G. McVey, A. Cross, A. P. Abbotts, and N. D. Stow.** 1997. The catalytic subunit of the DNA polymerase of herpes simplex virus type 1 interacts specifically with the C terminus of the UL8 component of the viral helicase-primase complex. J. Virol. **71:**6390–6397.
50. **McCarty, D. M., T. H. Ni, and N. Muzyczka.** 1992. Analysis of mutations in adeno-associated virus Rep protein in vivo and in vitro. J. Virol. **66:**4050–4057.
51. **McCarty, D. M., D. J. Pereira, I. Zolotukhin, X. Zhou, J. H. Ryan, and N. Muzyczka.** 1994. Identification of linear DNA sequences that specifically bind the adeno-associated virus Rep protein. J. Virol. **68:**4988–4997.
52. **McCarty, D. M., J. H. Ryan, S. Zolotukhin, X. Zhou, and N. Muzyczka.** 1994. Interaction of the adeno-associated virus Rep protein with a sequence within the A palindrome of the viral terminal repeat. J. Virol. **68:**4998–5006.
53. **McCarty, D. M., S. M. Young, Jr., and R. J. Samulski.** 2004. Integration of adeno-associated virus (AAV) and recombinant AAV vectors. Annu. Rev. Genet. **38:**819–845.
54. **McLean, C., A. Buckmaster, D. Hancock, A. Buchan, A. Fuller, and A. Minson.** 1982. Monoclonal antibodies to three non-glycosylated antigens of herpes simplex virus type 2. J. Gen. Virol. **63:**297–305.
55. **McLean, G. W., A. P. Abbotts, M. E. Parry, H. S. Marsden, and N. D. Stow.** 1994. The herpes simplex virus type 1 origin-binding protein interacts specifically with the viral UL8 protein. J. Gen. Virol. **75:**2699–2706.
56. **Muzyczka, N., and K. I. Berns.** 2001. Parvoviridae: the viruses and their replication, p. 2327–2346. *In* D. M. Knipe and P. M. Howley (ed.), Fields virology, 4th ed., vol. 2. Lippincott Williams & Wilkins, Philadelphia, PA.
57. **Nash, K., W. Chen, M. Salganik, and N. Muzyczka.** 2009. Identification of cellular proteins that interact with the adeno-associated virus rep protein. J. Virol. **83:**454–469.
58. **Nguyen, M. L., and J. A. Blaho.** 2007. Apoptosis during herpes simplex virus infection. Adv. Virus Res. **69:**67–97.
59. **Oehmig, A., C. Fraefel, and X. O. Breakefield.** 2004. Update on herpesvirus amplicon vectors. Mol. Ther. **10:**630–643.
60. **Owens, R. A., M. D. Weitzman, S. R. Kyostio, and B. J. Carter.** 1993. Identification of a DNA-binding domain in the amino terminus of adeno-associated virus Rep proteins. J. Virol. **67:**997–1005.
61. **Raj, K., P. Ogston, and P. Beard.** 2001. Virus-mediated killing of cells that lack p53 activity. Nature **412:**914–917. (Erratum, **416:**202, 2002.)
62. **Ryan, J. H., S. Zolotukhin, and N. Muzyczka.** 1996. Sequence requirements for binding of Rep68 to the adeno-associated virus terminal repeats. J. Virol. **70:**1542–1553.
63. **Sacks, W. R., C. C. Greene, D. P. Aschman, and P. A. Schaffer.** 1985. Herpes simplex virus type 1 ICP27 is an essential regulatory protein. J. Virol. **55:**796–805.
64. **Saeki, Y., C. Fraefel, T. Ichikawa, X. O. Breakefield, and E. A. Chiocca.** 2001. Improved helper virus-free packaging system for HSV amplicon vectors using an ICP27-deleted, oversized HSV-1 DNA in a bacterial artificial chromosome. Mol. Ther. **3:**591–601.
65. **Saudan, P., J. Vlach, and P. Beard.** 2000. Inhibition of S-phase progression by adeno-associated virus Rep78 protein is mediated by hypophosphorylated pRb. EMBO J. **19:**4351–4361.
66. **Schmidt, M., S. Afione, and R. M. Kotin.** 2000. Adeno-associated virus type 2 Rep78 induces apoptosis through caspase activation independently of p53. J. Virol. **74:**9441–9450.
67. **Schmidt, M., J. A. Chiorini, S. Afione, and R. Kotin.** 2002. Adeno-associated virus type 2 Rep78 inhibition of PKA and PRKX: fine mapping and analysis of mechanism. J. Virol. **76:**1033–1042.

68. **Schwartz, R. A., C. T. Carson, C. Schuberth, and M. D. Weitzman.** 2009. Adeno-associated virus replication induces a DNA damage response coordinated by DNA-dependent protein kinase. J. Virol. **83:**6269–6278.
69. **Shao, R. G., C. X. Cao, H. Zhang, K. W. Kohn, M. S. Wold, and Y. Pommier.** 1999. Replication-mediated DNA damage by camptothecin induces phosphorylation of RPA by DNA-dependent protein kinase and dissociates RPA: DNA-PK complexes. EMBO J. **18:**1397–1406.
70. **Shirata, N., A. Kudoh, T. Daikoku, Y. Tatsumi, M. Fujita, T. Kiyono, Y. Sugaya, H. Isomura, K. Ishizaki, and T. Tsurumi.** 2005. Activation of ataxia telangiectasia-mutated DNA damage checkpoint signal transduction elicited by herpes simplex virus infection. J. Biol. Chem. **280:**30336–30341.
71. **Slanina, H., S. Weger, N. D. Stow, A. Kuhrs, and R. Heilbronn.** 2006. Role of the herpes simplex virus helicase-primase complex during adeno-associated virus DNA replication. J. Virol. **80:**5241–5250.
72. **Smith, I. L., M. A. Hardwicke, and R. M. Sandri-Goldin.** 1992. Evidence that the herpes simplex virus immediate early protein ICP27 acts post-transcriptionally during infection to regulate gene expression. Virology **186:**74–86.
73. **Smith, R. H., and R. M. Kotin.** 2000. An adeno-associated virus (AAV) initiator protein, Rep78, catalyzes the cleavage and ligation of single-stranded AAV ori DNA. J. Virol. **74:**3122–3129.
74. **Smith, R. H., and R. M. Kotin.** 1998. The Rep52 gene product of adeno-associated virus is a DNA helicase with 3′-to-5′ polarity. J. Virol. **72:**4874–4881.
75. **Stracker, T. H., G. D. Cassell, P. Ward, Y. M. Loo, B. van Breukelen, S. D. Carrington-Lawrence, R. K. Hamatake, P. C. van der Vliet, S. K. Weller, T. Melendy, and M. D. Weitzman.** 2004. The Rep protein of adeno-associated virus type 2 interacts with single-stranded DNA-binding proteins that enhance viral replication. J. Virol. **78:**441–453.
76. **Taylor, T. J., M. A. Brockman, E. E. McNamee, and D. M. Knipe.** 2002. Herpes simplex virus. Front. Biosci. **7:**d752–d764.
77. **Timpe, J. M., K. C. Verrill, and J. P. Trempe.** 2006. Effects of adeno-associated virus on adenovirus replication and gene expression during coinfection. J. Virol. **80:**7807–7815.
78. **Trempe, J. P., E. Mendelson, and B. J. Carter.** 1987. Characterization of adeno-associated virus rep proteins in human cells by antibodies raised against rep expressed in Escherichia coli. Virology **161:**18–28.
79. **Tsukamoto, T., N. Hashiguchi, S. M. Janicki, T. Tumbar, A. S. Belmont, and D. L. Spector.** 2000. Visualization of gene activity in living cells. Nat. Cell Biol. **2:**871–878.
80. **Ward, P., M. Falkenberg, P. Elias, M. Weitzman, and R. M. Linden.** 2001. Rep-dependent initiation of adeno-associated virus type 2 DNA replication by a herpes simplex virus type 1 replication complex in a reconstituted system. J. Virol. **75:**10250–10258.
81. **Ward, P. L., and B. Roizman.** 1994. Herpes simplex genes: the blueprint of a successful human pathogen. Trends Genet. **10:**267–274. (Erratum, **10:**380.)
82. **Weindler, F. W., and R. Heilbronn.** 1991. A subset of herpes simplex virus replication genes provides helper functions for productive adeno-associated virus replication. J. Virol. **65:**2476–2483.
83. **Weitzman, M. D., K. J. Fisher, and J. M. Wilson.** 1996. Recruitment of wild-type and recombinant adeno-associated virus into adenovirus replication centers. J. Virol. **70:**1845–1854.
84. **Wilkinson, D. E., and S. K. Weller.** 2006. Herpes simplex virus type I disrupts the ATR-dependent DNA-damage response during lytic infection. J. Cell Sci. **119:**2695–2703.
85. **Wilkinson, D. E., and S. K. Weller.** 2005. Inhibition of the herpes simplex virus type 1 DNA polymerase induces hyperphosphorylation of replication protein A and its accumulation at S-phase-specific sites of DNA damage during infection. J. Virol. **79:**7162–7171.
86. **Wilkinson, D. E., and S. K. Weller.** 2004. Recruitment of cellular recombination and repair proteins to sites of herpes simplex virus type 1 DNA replication is dependent on the composition of viral proteins within prereplicative sites and correlates with the induction of the DNA damage response. J. Virol. **78:**4783–4796.
87. **Wonderling, R. S., and R. A. Owens.** 1997. Binding sites for adeno-associated virus Rep proteins within the human genome. J. Virol. **71:**2528–2534.
88. **Wu, J., M. D. Davis, and R. A. Owens.** 1999. Factors affecting the terminal resolution site endonuclease, helicase, and ATPase activities of adeno-associated virus type 2 Rep proteins. J. Virol. **73:**8235–8244.
89. **Yang, Q., F. Chen, J. Ross, and J. P. Trempe.** 1995. Inhibition of cellular and SV40 DNA replication by the adeno-associated virus Rep proteins. Virology **207:**246–250.
90. **Young, S. M., Jr., D. M. McCarty, N. Degtyareva, and R. J. Samulski.** 2000. Roles of adeno-associated virus Rep protein and human chromosome 19 in site-specific recombination. J. Virol. **74:**3953–3966.
91. **Zhou, X., I. Zolotukhin, D. S. Im, and N. Muzyczka.** 1999. Biochemical characterization of adeno-associated virus rep68 DNA helicase and ATPase activities. J. Virol. **73:**1580–1590.
92. **Zou, Y., Y. Liu, X. Wu, and S. M. Shell.** 2006. Functions of human replication protein A (RPA): from DNA replication to DNA damage and stress responses. J. Cell. Physiol. **208:**267–273.

6. Analysis of the Molecular Interactions between the AAV2 Rep Proteins and HSV-1 DNA Replication

Manuscript

AAV2 Rep68 can bind to consensus Rep-binding sites on the HSV-1 genome

Michael Seyffert[1], Daniel L. Glauser[1,2], Kurt Tobler[1], Oleg Georgiev[3], Rebecca Vogel[1], Bernd Vogt[1], Leticia Agúndez[4], Michael Linden[4], Hildegard Büning[5], Mathias Ackermann[1], and Cornel Fraefel[1]

[1]Institute of Virology, University of Zurich, Zurich, Switzerland;
[2]Suisselab AG, Zollikofen, Switzerland;
[3]Institute of Molecular Life Sciences, University of Zurich, Zurich, Switzerland;
[4]Department of Infectious Diseases, King's College London School of Medicine at Guy's, King's and St. Thomas Hospital, London, United Kingdom;
[5]Center for Molecular Medicine Cologne, University of Cologne, Cologne, Germany;

Running title: AAV2 Rep-binding sites on the HSV-1 genome

Manuscript in preparation

Own Contribution

- Cloning of plasmids
- Western Blots
- Replication assays
- Immuno-fluorescence
- Confocal microscopy
- Sequence analysis
- Electrophoretic mobility shift assays (EMSA)
- Chromatin immuno-precipitation assays (ChIP)
- qPCR
- Writing the manuscript
- Figure preparations

Short-Form Paper to Journal of Virology

AAV2 Rep68 can bind to consensus Rep-binding sites on the HSV-1 genome

Michael Seyffert[1], Daniel L. Glauser[1,2], Kurt Tobler[1], Oleg Georgiev[3], Rebecca Vogel[1], Bernd Vogt[1], Leticia Agúndez[4], Michael Linden[4], Hildegard Büning[5], Mathias Ackermann[1], and Cornel Fraefel[1]

[1]Institute of Virology, University of Zurich, Zurich, Switzerland;
[2]Suisselab AG, Zollikofen, Switzerland;
[3]Institute of Molecular Life Sciences, University of Zurich, Zurich, Switzerland;
[4]Department of Infectious Diseases, King's College London School of Medicine at Guy's, King's and St. Thomas Hospital, London, United Kingdom;
[5]Center for Molecular Medicine Cologne, University of Cologne, Cologne, Germany;

Running title: AAV2 Rep-binding sites on the HSV-1 genome

#To whom correspondence should be addressed:
Cornel Fraefel
Institute of Virology, University of Zurich,
Winterthurerstrasse 266a,
CH-8057 Zurich, Switzerland
Phone: (41) 44 6358713
Fax: (41) 44 6358911
E-mail: cornel.fraefel@vetvir.uzh.ch

Word count:
Abstract: 131
Text: 2495
Number of figures: 3
Number of supplementary materials: 1

ABSTRACT

Adeno-associated virus type 2 (AAV2) has previously been shown to inhibit the replication of its helper virus herpes simplex virus type 1 (HSV-1). The inhibitory activity has been linked to the expression of the AAV2 Rep proteins, more precisely, the helicase and DNA binding domains of Rep68 and Rep78, but the exact mechanism of inhibition is not known. Here, we show that the AAV2 Rep68 protein can bind to consensus Rep-binding sites (RBS) on the HSV-1 genome and that the helicase activity of Rep68 can prevent the replication of any DNA it binds to. These results support our hypothesis that the mechanism of Rep68/78 mediated inhibition of HSV-1 DNA replication may involve direct binding of Rep proteins to the HSV-1 genome and modification of the bound DNA via the helicase activity.

Keywords: AAV2; HSV-1; Rep-binding site; Rep helicase.

FINDINGS

Adeno-associated virus type 2 (AAV2) is a nonpathogenic human parvovirus with a unique biphasic life cycle. In the absence of a helper virus, AAV2 establishes a latent infection while in the presence of a helper virus, such as adenovirus (Ad), herpes simplex virus type 1 (HSV-1) or papillomavirus, it undergoes lytic replication (5, 16, 41, 44). The AAV2 genome is a single-stranded DNA (ssDNA) of 4,680 nucleotides, which is packaged into an icosahedral capsid with a diameter of 20 nm (26). The genome harbors two clusters of genes, *rep* and *cap*, which are both flanked by inverted terminal repeats (ITRs). The ITRs form hairpin structures and contain a Rep-binding site (RBS) and a terminal resolution site (TRS), which together act as viral origin of DNA replication (17, 38). The *cap* gene is transcribed from the p40 promoter and encodes the capsid proteins VP1, VP2, and VP3, which differ in their N termini due to alternative start codons (2, 6). The *rep* gene encodes the Rep proteins, which are expressed in four different forms due to transcription from two different promoters, p5 and p19, and alternative splicing at an intron near the C-terminal end (7). The different Rep proteins are termed Rep40, Rep52, Rep68, and Rep78 according to their apparent molecular weight. The Rep proteins are involved in diverse processes in the viral life cycle, such as DNA replication, regulation of gene expression, genome packaging, and site-specific genomic integration (18, 20, 23, 24, 35).

The minimal set of HSV-1 proteins required for initiating AAV2 replication consists of UL5, UL8, and UL52, which together form the HSV-1 helicase/primase complex, as well as the ssDNA binding protein ICP8 (UL29) (1, 42, 43). However, the HSV-1 immediate-early proteins ICP4 and ICP0, the HSV-1 polymerase (UL42 and UL30) and the US12 gene product, strongly enhance AAV2 replication (1). AAV2 has developed strategies to inhibit helper virus replication, likely to reduce competition (12, 13, 15, 19, 21, 30, 39). For example, expression of the AAV2 non-structural proteins Rep68/78 initiates a complete shut-off of HSV-1 DNA replication (12, 13). In a previous study we demonstrated that the AAV2 Rep protein domains responsible for the inhibition of HSV-1 DNA replication include the DNA-binding and the ATPase/helicase domains

in *cis*, while the endonuclease activity is not required (12). We also showed that Rep-mediated inhibition of HSV-1 occurs also in absence of AAV2 DNA and is not due to alterations of HSV-1 immediate-early (IE) and early (E) gene expression, nor is it due to the Rep-mediated induction of toxic stress in the cell, but rather occurs at the stage of HSV-1 DNA replication itself. We therefore hypothesized that a possible mechanism of Rep68/78 mediated inhibition of HSV-1 DNA replication may involve binding of Rep proteins to consensus Rep-binding sites (RBS) on the HSV-1 genome and modification of the bound DNA substrate via the Rep helicase activity, in particular if there was no TRS in close proximity to the RBS that together would act as an origin of DNA replication. To investigate this possibility, we now addressed the following two questions: (i) can the AAV2 Rep68/78 helicase activity in absence of the endonuclease activity inhibit replication of any DNA when binding is facilitated? (ii) Do consensus RBS exist on the HSV-1 genome and if so, can AAV2 Rep68/78 bind to these sites?

To address the first question, we used an assay that we have established previously to investigate the replication of HSV-1 and AAV2 DNA both in live and in fixed cells (10, 11, 13). Briefly, cells are co-transfected with a plasmid that contains 40 binding sites for the lac repressor protein LacI and an HSV-1 origin of DNA replication (pHSV-lacO), and a second plasmid that encodes the *lac*-operon binding motif of LacI fused with an autofluorescent protein, such as enhanced yellow fluorescent protein (EYFP-LacI). In presence of HSV-1 helper functions, pHSV-lacO replicons (and therefore the binding sites for LacI) are amplified (because of the HSV-1 origin of DNA replication) and recruitment of autofluorescent LacI protein then allows visualization of pHSV-lacO DNA replication. We have now modified this assay by transfecting a third plasmid encoding the AAV2 Rep52 protein, which contains the helicase domain but not the DNA binding or endonuclease domains of Rep68/78, fused with the *lac*-operon binding motif of LacI (Rep52-LacI) (Fig. 1 A and B). This modified Rep52 protein is prone to bind to *lac*-operon binding sequences present on the pHSV-lacO replicon and therefore allowed us to study the Rep helicase activity in absence of the endonuclease activity and independent of the interaction

between the AAV2 Rep DNA-binding domain and the RBS. The results shown in Fig. 1 (C and D) demonstrate that Rep52-LacI was indeed able to strongly inhibit the replication of pHSV-lacO, as cells containing mature stage IV RCs were not observed. By contrast, in cultures expressing Rep52, which lacks a DNA binding domain, or Rep52K340H-LacI, which has a point mutation that inactivates the helicase activity (8, 25) and is fused to the DNA binding domain of LacI, 28.3±7.6% or 14.7±6.1% of stage IV RCs were observed, respectively. Also, in the cultures transfected with the Rep52-LacI encoding plasmid the frequency of stage III RCs was reduced by approximately 50% while the frequency of cells showing diffuse EYFP-fluorescence (stage I; no RCs) was much higher when compared with the control cultures (no Rep, Rep52 and Rep52K340H-LacI). The distribution of stage I-IV RCs was comparable in cells transfected with the Rep52 and the Rep52K340H-LacI encoding plasmids, indicating that Rep52-LacI competition with EYFP-LacI did not interfere with detection of RCs. Moreover, while Rep68/78 can efficiently inhibit HSV-1 DNA replication (12, 13), transfection of Rep52, Rep52-LacI, and Rep52K340H-LacI encoding plasmids did not affect HSV-1 DNA replication (data not shown). These results indicate that the AAV2 Rep helicase activity in absence of the Rep DNA binding and endonuclease activities can inhibit the replication of any DNA template as long as it can bind to the DNA. Of note, we have observed 5.3±9.2% and 17.3±4.6% of cells showing distinct foci when transfected with Rep52-LacI or the helicase mutant Rep52K340H-LacI respectively, which were not observed when transfected with the Rep52 or the empty backbone plasmid pcDNA3.1+. We considered this pattern as an artefact occurring from overexpression from the reporter plasmids.

We addressed the second question, whether AAV2 Rep can bind to putative RBS on the HSV-1 genome, initially by screening the HSV-1 (strain F) genome for the minimal consensus RBS motif GAGYGAGC as a prerequisite for the ability of Rep to bind to dsDNA templates via its DNA-binding domain (9). Such sites are indeed present and are shown in Fig. 2. All consensus RBS are located within coding sequences of genes found in the unique long (U_L) segment of the

HSV-1 genome (Fig.2 A). Sequence alignment revealed that all putative HSV-1 RBS consist of two complete GAGC repeats with the exception of RBS No. 7, which contains a T in place of a C but still complies with the GAGYGAGC consensus sequence (Fig.2 B). Moreover, the regions upstream of the RBS-motif seem to be more conserved than the downstream regions (Fig.2 B). No consensus TRS (CCAACT) (18, 38) was found near the putative HSV-1 RBS. We next tested the capability of AAV2 Rep proteins to bind via the DNA-binding domain to putative HSV-1 RBS by electrophoretic mobility-shift assays (EMSA) using purified His-tagged Rep68 proteins. We designed 37-mer duplexed oligonucleotides harbouring selected putative RBS (No. 1-5), which were radioactively labelled with [y-^{32}P]ATP. An oligonucleotide containing a random sequence from within the UL44 gene harbouring no putative RBS was used as the negative control. An oligonucleotide containing the RBS from the AAV2 inverted terminal repeat (ITR) was used as the positive control (Fig. 2 B). Approximately 5 fmol of each duplexed oligonucleotide was incubated with 0, 120, or 240 ng of Rep68 protein for 30' at room temperature (RT) and then subjected to polyacrylamide gel electrophoresis. After 3h, the gel was dried and exposed to Fujifilm Imaging Plates, which were developed with a Fujifilm FLA-7000 Image Plate reader. We observed a dose-dependent shift from the unbound DNA template to the same level as the bound positive control (ITR) for every oligonucleotide (No. 1-5) examined (Fig. 3 A). As expected, the negative control (nc) did not show any bands at the level of the shifted DNA. Of note, the area of the shifted oligonucleotides (No. 1-5 as well as ITR) is characterized by multiple bands with different sizes. This may be due to the different oligomerization states Rep68 can form on ds DNA (28, 45, 46). To confirm that binding of Rep68 to the putative RBS is indeed specific, we performed EMSAs as described above, except that 5 fmol of non-labeled duplex ITR oligonucleotide was used as competitor. The ITR competitor DNA appeared to inhibit binding of Rep to the putative RBS oligonucleotides, as no shift was observed under these conditions (Fig.3 B). In order to investigate whether AAV2 Rep68 is able to bind to the consensus RBS on the HSV-1 genome also in HSV-1 infected cells, we performed chromatin-immunoprecipitation

(ChIP) assays. For this, Vero cells were transfected with plasmids expressing either the Rep68/78, or Rep52 proteins fused with enhanced green fluorescent protein (EGFP). The next day, the cells were infected with wt HSV-1 (strain F) at an MOI of 40. At 16 h after infection, the cells were fixed with 4% paraformaldehyde (PFA), sonicated for 10min (at 25% amplitude and duty cycle 10 on ice) and processed for ChIP using the GFP-Trap® kit (Chromotek). The immunoprecipitated DNA was then analyzed by quantitative (q)PCR using primers specific for the consensus RBS No. 1-5 (Suppl. Table. 1). Primers for amplification of a sequence from the HSV-1 genome harbouring no proximal RBS (U_S1) served as a negative control (Suppl. Table. 1). The qPCR mix was the following: 0.25µl of each primer [10µM], 10µl of SYBR® Green mix (SYBR® Green PCR Master Mix, Applied Biosystems) and 2.5µl of DNA in a final volume of 20µl; The reaction was carried out as follows: 95°C for 3min, 39 cycles of 95°C for 15sec and 60°C for 1min followed by a final elongation step at 95°C for 10min. The data was analyzed using the percent input method (as described by Life Technologies™) in which the signal from the qPCR reaction with the primers specific for the U_S1 sequence (negative control) served as the background and was set as 1. All other values were then normalized to the U_S1 value. The results are shown in Fig. 3C and can be summarized as follows: The AAV2 Rep68/78-GFP fusion protein is capable of binding to all HSV-1 consensus RBSs tested. Although there appears to be some variation in the affinities between the RBS No. 1-5 sequences, the values were all significantly higher than the value obtained with the U_S1 primers (no pRBS). On the other hand, the Rep52-GFP protein, which lacks the RBS binding domain, did not appear to bind to any of the RBS tested (data not shown). These results indicate that AAV2 Rep68/78 is indeed capable of interacting with the HSV-1 genome via its RBS binding domain.

In this report we demonstrated that the AAV2 Rep68 proteins are capable of binding to pRBS on the HSV-1 ds DNA genome *in silico*, *in vitro*, and in HSV-1 infected cells. Our EMSA and ChIP data revealed that binding of Rep68 to consensus RBS is specific and requires the Rep DNA-binding domain. Moreover, Rep68 is not able to bind directly to random HSV-1 dsDNA

sequences (UL44 and US1) that do not contain a putative RBS. In addition, we showed that the Rep helicase activity can inhibit replication of any DNA substrate it can bind to.

Replicative stress is rapidly sensitized in the cell by numerous stress response factors and pathways such as DNA-damage response (DDR) or DNA-damage tolerance (DDT) (Reviewed in (47)). One of the best understood stress responses resembles the DDR pathway initiated by the sensor-kinase ataxia-telangiectasia mutated (ATM)- and Rad3-related (ATR), which is activated upon stalling of the replication fork (RF). At a stalled RF, the helicase activity of the mini-chromosome maintenance protein (MCM2) is uncoupled from the replication complex and continues unwinding, which is generating a stretch of ssDNA recognized and covered by the replication protein A (RPA) (32–34). This leads to the activation of ATR, which then induces a DDR resulting in cell-cycle arrest allowing the cell to resolve the stalled RF (4, 29, 31, 48, 49). However, persisting replication stress can result in the collapse of the RF, associated with double-strand breaks (DSBs) and the consecutive inhibition of DNA replication (14, 27).

We therefore hypothesize that binding of Rep to consensus RBS and the following helicase activity mimick a stalled RF, in particular when the Rep helicase activity is uncoupled from the replication activity, either because of the absence of a functional TRS or the absence of the endonuclease activity. Therefore it is likely that Rep-mediated unwinding of dsDNA is inducing a DDR which is similar to the one induced by a stalled RF. Support for this theory comes from previous observations that the AAV2 Rep68/78 proteins induce a DDR which is characterized by the activation of RPA and ATM, leading to a S-phase arrest (3, 12, 36, 37). However, in these studies the authors explain the activation of ATM as a response to nicks which were induced by Rep at TRSs located on the cellular chromatin. In contrast to this theory, we suggest that the activation of ATM might be due to Rep-induced dsDNA breaks caused by persistend stalling RFs. Indications for this theory comes from the simple fact that the existence of a putative RBS with a TRS in close proximity on the cellular genome is much less likely than a putative RBS without a functional TRS in close proximity.

If Rep indeed is inducing a stalled RF remains to be elucidated. Also, if the Rep helicase is indeed unwinding the HSV-1 DNA is a matter of current investigations. However, we demonstrate that the Rep helicase activity is inhibiting replication of dsDNA templates it can bind to and that Rep is indeed capable of binding to the HSV-1 genome at multiple consensus RBS.

FIGURE LEGENDS

Figure 1. Inhibition of DNA replication by the AAV2 Rep helicase activity. (A) Schematic representation of the Rep constructs analyzed in this experiment. The functional domains of interest for this study are indicated. (B) Western analysis of Rep52, Rep52-LacI, and Rep52K340H-LacI. Vero cells (200'000) were transfected with 0.1µg of plasmids encoding Rep52, Rep52-LacI, or Rep52K340H-LacI, or the empty plasmid backbone pcDNA3.1+ (no Rep). After 24h the cells were harvested and processed for Western blotting using a Rep-specific antibody (ms anti Rep-mAb, clone 303.9, Fitzgerald; 1:100). Actin staining served as loading-control. (C) HSV-1 DNA replication assay. Vero cells (150'000) were co-transfected with 0.05µg of the HSV-1 replicon plasmid pHSV-LacO, 0.01µg of the reporter plasmid pSV2EYFPLacI and 0.05µg of a plasmid encoding either Rep52-LacI, the helicase deficient mutant Rep52K340H-LacI, wtRep52 or the empty plasmid backbone pcDNA3.1+ (no Rep). One day after transfection the cells were superinfected with wt HSV-1 (strain F) at an MOI of 5. After 16 h, the cells were fixed with 4% paraformaldehyde (PFA), immunostained for Rep with a primary mouse (ms) anti Rep-mAb (clone 303.9, Fitzgerald, 1:100) and a secondary goat (gt) anti-ms IgG(H+L)–Alexa Fluor 594 (Molecular Probes®, 1:500) (red insets), and then subjected to confocal laser scanning microscopy (SP2 CLSM, Leica). The numbers of cells displaying pHSV-lacO RCs at stage I, II, III or IV, as well as the number of cells showing foci were determined. (C) Percentage of cells containing stage I, II, III, or IV RCs or foci evaluated from experiments shown in panel B. Error bars represent standard deviation (SD) from three independent experiments with 80-100 cells counted in each experiment. Asterisks indicate statistically significant differences based on a paired two-tail students t-test (* = $p<0.05$; ** = $p<0.01$).

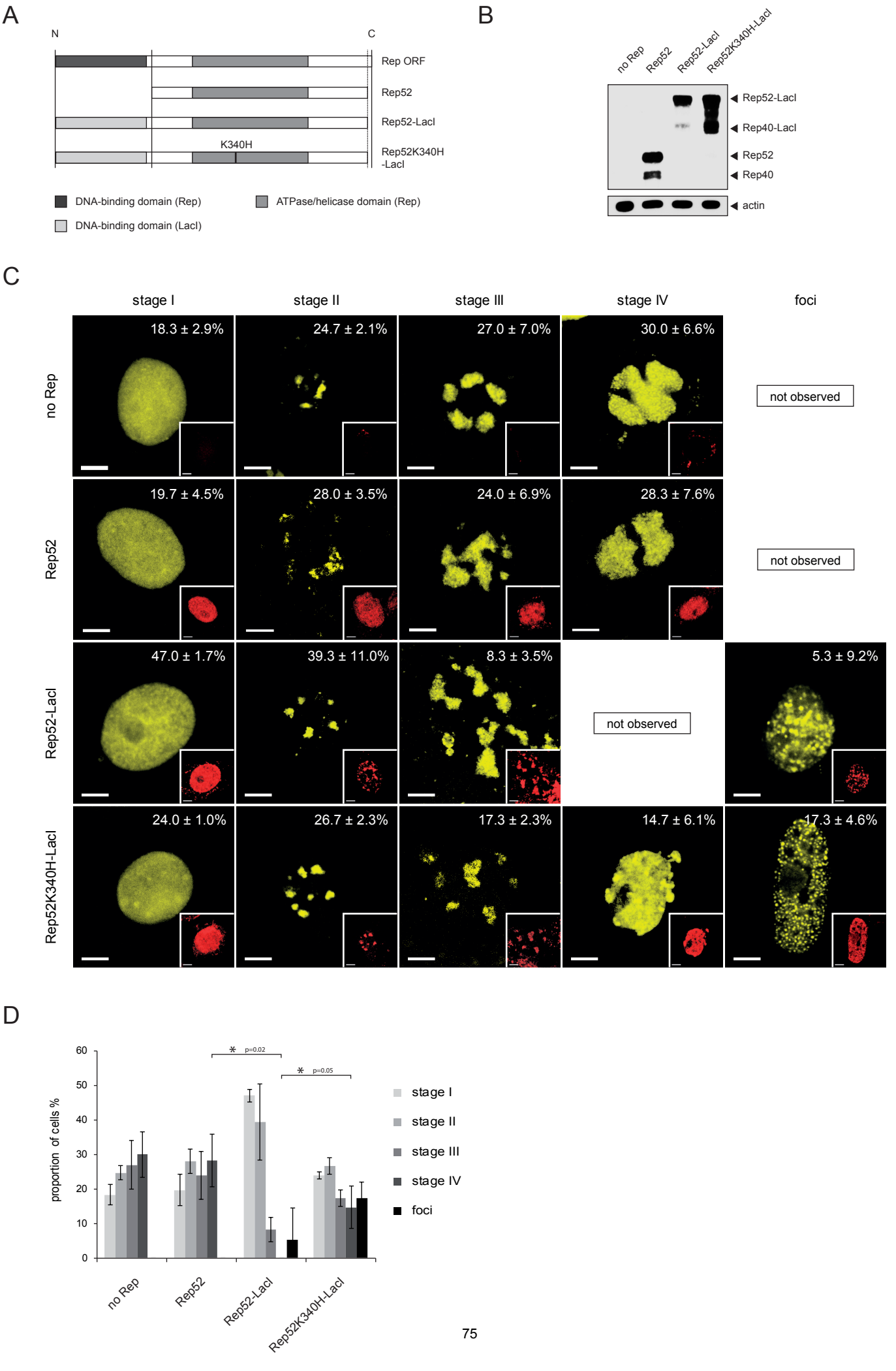
Figure 2. The HSV-1 genome contains nine putative Rep-binding sites. (A) The minimal AAV2 Rep-binding site motif GAGYGAGC was used to identify consensus Rep-binding sites within the HSV-1 (strain F) genome. The arrowheads facing down represent RBS located on the

plus-strand and the arrowheads facing up represent RBS located on the minus-strand. The RBS were numbered randomly from 1 to 9 as indicated. The HSV-1 genes harbouring a consensus RBS are highlighted. (B) Alignment of the minimal RBS motif GAGYGAGC (grey box), the AAV2 ITR RBS, the nine putative HSV-1 RBS identified in panel A, and the negative control sequence (nc) within the UL44 gene. The illustration was generated using the *prettyplot* function of the online tool EMBOSS (http://pro.genomics.purdue.edu/emboss/) and represents the sequences of the oligonucleotides used for the electrophoretic mobility shift assays (EMSA; see Fig.3). Conserved nucleotides upstream and downstream of the GAGYGAGC motif are indicated with asterisks. The numbers on the right indicate the exact nucleotide (nt) position within the AAV2 genome (ITR) or the HSV-1 genome (No. 1-9 and nc).

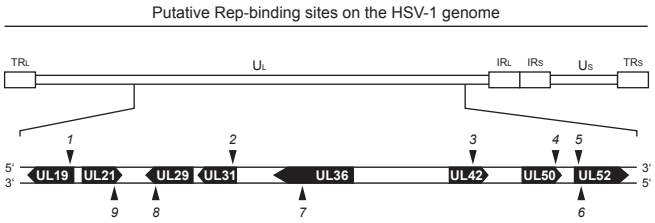
Figure 3. Rep-binding assays. (A) Electrophoretic mobility shift assays (EMSA) were performed with purified Rep68 proteins and radiolabeled duplex oligonucleotides No. 1-5 harbouring putative RBS, the negative control (nc) or the positive control (ITR) (Fig.2 B, bold numbers). The amount (ng) of Rep68 protein used is indicated at the top of the blot. (B) EMSA competition assays were performed with cold competitor DNA consisting of the positive control oligonucleotide (ITR). (C) Chromatin immuno-precipitation (ChIP) assays were performed with Vero cells (approx. $1.5x10^6$ cells in a 6cm cell culture dish) transfected with 0.5µg of a plasmid encoding the Rep68-EGFP fusion protein (Rep68-GFP) and superinfected with wtHSV-1 (strain F) at an MOI of 40. At 16 h after infection the cells were processed for ChIP (as described in the text). The precipitated DNA was analyzed by qPCR using specific primers flanking the putative RBS No. 1-5 on the HSV-1 genome or primers flanking a sequence of the HSV-1 genome that contains no proximal putative RBS (no RBS) (see Supplementary Table.1). The data was quantified using the percentage input method (as described in the text) and the values were normalized to the *no RBS*-control, which was set as 1. Bars represent mean values and standard errors (SE) from 3-6 individual experiments. Asterisks indicate statistically significant

differences between the negative control (no RBS) and the putative RBS based on a paired two-tail students t-test (* = $p<0.05$; ** = $p<0.01$).

Supplementary Table 1. Primer pairs used for the ChIP assay. Forward and reverse primer sequences used for qPCR of precipitated DNA are listed. Primers were generated using the online tool Primer3® (22, 40). The efficiencies for all primer pairs were tested with serial dilutions of purified HSV-1 DNA (strain F) under the same qPCR conditions as used in Fig.3C. This data was used to standardize the ChIP protocol by including the efficiency coefficient for each primer pair in the percentage input (%-Input) method calculations.


A
N
C
Rep ORF
Rep52
Rep52-LacI
K340H
Rep52K340H
-LacI
DNA-binding domain (Rep)
ATPase/helicase domain (Rep)
DNA-binding domain (LacI)
B
no Rep
Rep52
Rep52-LacI
Rep52K340H-LacI
Rep52-LacI
Rep40-LacI
Rep52
Rep40
actin
C
stage I
stage II
stage III
stage IV
foci
no Rep
Rep52
Rep52-LacI
Rep52K340H-LacI
18.3 ± 2.9%
24.7 ± 2.1%
27.0 ± 7.0%
30.0 ± 6.6%
not observed
19.7 ± 4.5%
28.0 ± 3.5%
24.0 ± 6.9%
28.3 ± 7.6%
not observed
47.0 ± 1.7%
39.3 ± 11.0%
8.3 ± 3.5%
not observed
5.3 ± 9.2%
24.0 ± 1.0%
26.7 ± 2.3%
17.3 ± 2.3%
14.7 ± 6.1%
17.3 ± 4.6%
D
proportion of cells %
60
50
40
30
20
10
0
p=0.02
p=0.05
stage I
stage II
stage III
stage IV
foci
no Rep
Rep52
Rep52-LacI
Rep52K340H-LacI

A

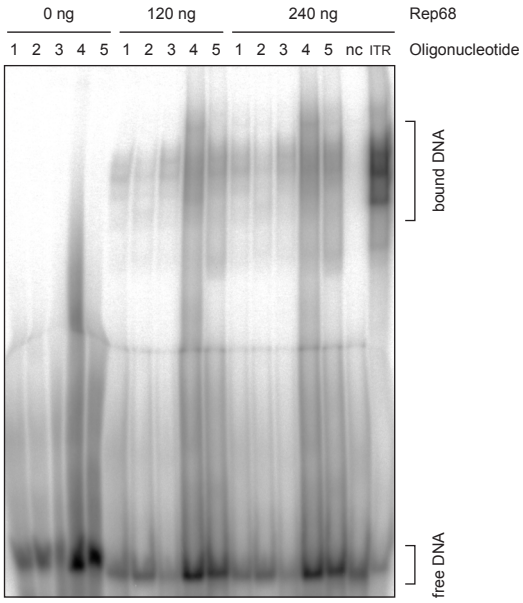


B

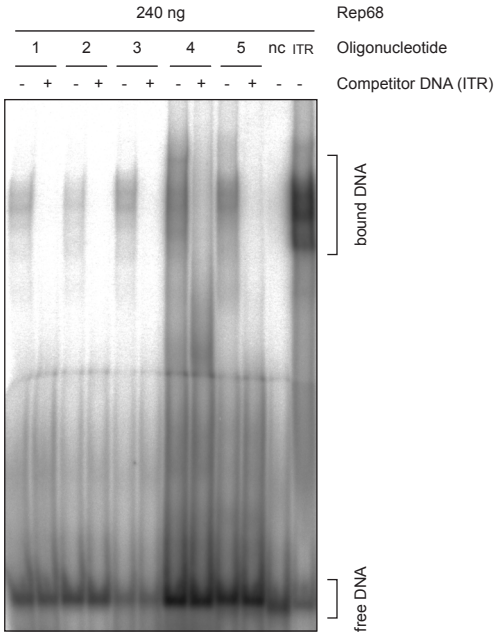
Consensus RBS sequence alignment (5' to 3')

No.		Position (nt)
nc	C G T C T G G C C C G C G G G G A C C A C C A T C A C T T C C A C C G G A	45418-45454
ITR	G G C G G C C T C A G T **G A G C G A G C** G A G C G C G C A G A G A G G G A	81-117
1	C C T G G A C G T T G C **G A G C G A G C** T G T T G G A T G G C G C G C A A	39936-39972
2	C A G G G G C A C C G A **G A G C G A G C** G C A C G A T G G C G A T C T C C	67040-67076
3	C C G G G C C T C T G G **G A G C G A G C** C A A C A G A C A G C C A G G A C	94024-94060
4	G G A G G C C C C C C C C **G A G C G A G C** G C G G G A C C G G G G G T T T T	107968-108004
5	G T C T G T C G C T T C **G A G C G A G C** G G A C G A C G T C G C C G C G C	109287-109323
6	C G T G C G C C C C A C **G A G C G A G C** G T A G G G A C G C G C C C T G A	109529-109493
7	A G G C G T A C C T G C **G A G T G A G C** G G A G G C C C G G G G C C C C T	74589-74553
8	C C C C A A C A A C G T **G A G C G A G C** T G G C G A T G T A C T A C A T G	59318-59282
9	T G T C C G T G T T T G **G A G C G A G C** G A G A C G A A C G G T A A G A A	43587-43551

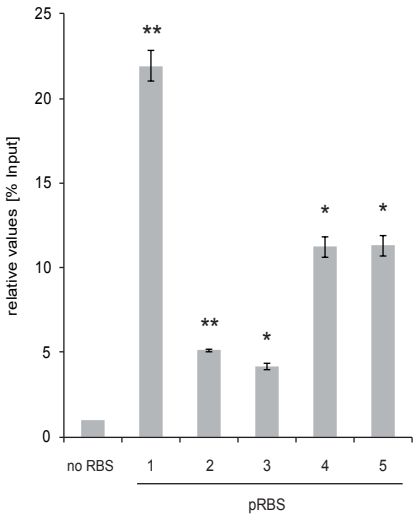
A


0 ng
120 ng
240 ng
Rep68
1 2 3 4 5 1 2 3 4 5 1 2 3 4 5 nc ITR
Oligonucleotide
bound DNA
free DNA

B


240 ng
Rep68
1 2 3 4 5 nc ITR
Oligonucleotide
- + - + - + - + - + - -
Competitor DNA (ITR)
bound DNA
free DNA

C


relative values [% Input]
25
20
15
10
5
0
**
**
*
*
*
no RBS
1
2
3
4
5
pRBS

Supplementary Table 1. primer pairs used for ChIP assay

#	forward	reverse
no RBS	gcttccttgtttggagacca	gtccagtcaaactccccaaa
1	cacgtgcagcatctggtc	cgagcaacccacacacaat
2	atcgtgttgatctgctgcac	gaggagatcgccatcgtg
3	ccctcaagttcttcctcacg	ggagtcctggctgtctgttg
4	caaagcgcttcgaaactacc	gggtgtgatagacccacagg
5	gctaaatggcgactccttcc	cgatgtggtccggttgtag

REFERENCES

1. **Alazard-Dany, N., A. Nicolas, A. Ploquin, R. Strasser, A. Greco, A. L. Epstein, C. Fraefel, and A. Salvetti.** 2009. Definition of herpes simplex virus type 1 helper activities for adeno-associated virus early replication events. PLoS Pathog. **5**:e1000340. doi:10.1371/journal.ppat.1000340.
2. **Becerra, S. P., F. Koczot, P. Fabisch, and J. A. Rose.** 1988. Synthesis of adeno-associated virus structural proteins requires both alternative mRNA splicing and alternative initiations from a single transcript. J Virol **62**:2745–2754.
3. **Berthet, C., K. Raj, P. Saudan, and P. Beard.** 2005. How adeno-associated virus Rep78 protein arrests cells completely in S phase. Proceedings of the National Academy of Sciences of the United States of America **102**:13634–13639.
4. **Boddy, M. N., B. Furnari, O. Mondesert, and P. Russell.** 1998. Replication checkpoint enforced by kinases Cds1 and Chk1. Science **280**:909–912.
5. **Buller, R. M., J. E. Janik, E. D. Sebring, and J. A. Rose.** 1981. Herpes simplex virus types 1 and 2 completely help adenovirus-associated virus replication. Journal of Virology **40**:241–247.
6. **Cassinotti, P., M. Weitzand, and J. D. Tratschin.** 1988. Organization of the adeno-associated virus (AAV) capsid gene: mapping of a minor spliced mRNA coding for virus capsid protein. Virology **167**:176–184.
7. **Chejanovsky, N., and B. J. Carter.** 1989. Mutagenesis of an AUG codon in the adeno-associated virus rep gene: effects on viral DNA replication. Virology **173**:120–128.
8. **Chejanovsky, N., and B. J. Carter.** 1990. Mutation of a consensus purine nucleotide binding site in the adeno-associated virus rep gene generates a dominant negative phenotype for DNA replication. J. Virol. **64**:1764–1770.

9. **Chiorini, J. A., S. M. Wiener, R. A. Owens, S. R. Kyostio, R. M. Kotin, and B. Safer.** 1994. Sequence requirements for stable binding and function of Rep68 on the adeno-associated virus type 2 inverted terminal repeats. Journal of Virology **68**:7448–7457.

10. **Fraefel, C., A. G. Bittermann, H. Bueler, I. Heid, T. Bachi, and M. Ackermann.** 2004. Spatial and temporal organization of adeno-associated virus DNA replication in live cells. Journal of Virology **78**:389–398.

11. **Glauser, D. L., O. Saydam, N. A. Balsiger, I. Heid, R. M. Linden, M. Ackermann, and C. Fraefel.** 2005. Four-dimensional visualization of the simultaneous activity of alternative adeno-associated virus replication origins. Journal of Virology **79**:12218–12230.

12. **Glauser, D. L., M. Seyffert, R. Strasser, M. Franchini, A. S. Laimbacher, C. Dresch, A. P. de Oliveira, R. Vogel, H. Büning, A. Salvetti, M. Ackermann, and C. Fraefel.** 2010. Inhibition of herpes simplex virus type 1 replication by adeno-associated virus rep proteins depends on their combined DNA-binding and ATPase/helicase activities. J. Virol. **84**:3808–3824. doi:10.1128/JVI.01503-09.

13. **Glauser, D. L., R. Strasser, A. S. Laimbacher, O. Saydam, N. Clément, R. M. Linden, M. Ackermann, and C. Fraefel.** 2007. Live covisualization of competing adeno-associated virus and herpes simplex virus type 1 DNA replication: molecular mechanisms of interaction. Journal of Virology **81**:4732–4743.

14. **Hanada, K., M. Budzowska, S. L. Davies, E. van Drunen, H. Onizawa, H. B. Beverloo, A. Maas, J. Essers, I. D. Hickson, and R. Kanaar.** 2007. The structure-specific endonuclease Mus81 contributes to replication restart by generating double-strand DNA breaks. Nat Struct Mol Biol **14**:1096–1104.

15. **Heilbronn, R., A. Burkle, S. Stephan, and H. Zur Hausen.** 1990. The adeno-associated virus rep gene suppresses herpes simplex virus-induced DNA amplification. Journal of Virology **64**:3012–3018.

16. **Hoggan, M. D., N. R. Blacklow, and W. P. Rowe.** 1966. Studies of small DNA viruses found in various adenovirus preparations: physical, biological, and immunological characteristics. Proceedings of the National Academy of Sciences of the United States of America **55**:1467–1474.

17. **Hong, G., P. Ward, and K. I. Berns.** 1992. In vitro replication of adeno-associated virus DNA. Proc. Natl. Acad. Sci. U.S.A. **89**:4673–4677.

18. **Im, D. S., and N. Muzyczka.** 1990. The AAV origin binding protein Rep68 is an ATP-dependent site-specific endonuclease with DNA helicase activity. Cell **61**:447–457.

19. **Jing, X. J., V. Kalman-Maltese, X. Cao, Q. Yang, and J. P. Trempe.** 2001. Inhibition of adenovirus cytotoxicity, replication, and E2a gene expression by adeno-associated virus. Virology **291**:140–151.

20. **King, J. A.** 2001. DNA helicase-mediated packaging of adeno-associated virus type 2 genomes into preformed capsids. The EMBO Journal **20**:3282–3291.

21. **Kleinschmidt, J. A., M. Mohler, F. W. Weindler, and R. Heilbronn.** 1995. Sequence elements of the adeno-associated virus rep gene required for suppression of herpes-simplex-virus-induced DNA amplification. Virology **206**:254–262.

22. **Koressaar, T., and M. Remm.** 2007. Enhancements and modifications of primer design program Primer3. Bioinformatics **23**:1289–1291.

23. **Kotin, R. M., R. M. Linden, and K. I. Berns.** 1992. Characterization of a preferred site on human chromosome 19q for integration of adeno-associated virus DNA by non-homologous recombination. EMBO Journal **11**:5071–5078.

24. **Kotin, R. M., J. C. Menninger, D. C. Ward, and K. I. Berns.** 1991. Mapping and direct visualization of a region-specific viral DNA integration site on chromosome 19q13-qter. Genomics **10**:831–834.

25. **Kyostio, S. R., R. S. Wonderling, and R. A. Owens.** 1995. Negative regulation of the adeno-associated virus (AAV) P5 promoter involves both the P5 rep binding site and the consensus ATP-binding motif of the AAV Rep68 protein. Journal of Virology **69**:6787–6796.

26. **Linden, R. M., and K. I. Berns.** 2000. Molecular biology of adeno-associated viruses, vol. 4.

27. **Lopes, M., M. Foiani, and J. M. Sogo.** 2006. Multiple mechanisms control chromosome integrity after replication fork uncoupling and restart at irreparable UV lesions. Mol Cell **21**:15–27.

28. **Mansilla-Soto, J., M. Yoon-Robarts, W. J. Rice, S. Arya, C. R. Escalante, and R. M. Linden.** 2009. DNA structure modulates the oligomerization properties of the AAV initiator protein Rep68. PLoS Pathog. **5**:e1000513. doi:10.1371/journal.ppat.1000513.

29. **Martinho, R. G., H. D. Lindsay, G. Flaggs, A. J. DeMaggio, M. F. Hoekstra, A. M. Carr, and N. J. Bentley.** 1998. Analysis of Rad3 and Chk1 protein kinases defines different checkpoint responses. EMBO J **17**:7239–7249.

30. **Nada, S., and J. P. Trempe.** 2002. Characterization of adeno-associated virus rep protein inhibition of adenovirus E2a gene expression. Virology **293**:345–355.

31. **Namiki, Y., and L. Zou.** 2006. ATRIP associates with replication protein A-coated ssDNA through multiple interactions. Proc. Natl. Acad. Sci. U.S.A. **103**:580–585.

32. **Nedelcheva, M. N., A. Roguev, L. B. Dolapchiev, A. Shevchenko, H. B. Taskov, A. Shevchenko, A. F. Stewart, and S. S. Stoynov.** 2005. Uncoupling of unwinding from DNA synthesis implies regulation of MCM helicase by Tof1/Mrc1/Csm3 checkpoint complex. J. Mol. Biol. **347**:509–521.

33. **Nitani, N., C. Yadani, H. Yabuuchi, H. Masukata, and T. Nakagawa.** 2008. Mcm4 C-terminal domain of MCM helicase prevents excessive formation of single-stranded DNA at stalled replication forks. Proc. Natl. Acad. Sci. U.S.A. **105**:12973–12978.

34. **Pacek, M., A. V. Tutter, Y. Kubota, H. Takisawa, and J. C. Walter.** 2006. Localization of MCM2-7, Cdc45, and GINS to the site of DNA unwinding during eukaryotic DNA replication. Mol. Cell **21**:581–587.

35. **Samulski, R. J., X. Zhu, X. Xiao, J. D. Brook, D. E. Housman, N. Epstein, and L. A. Hunter.** 1991. Targeted integration of adeno-associated virus (AAV) into human chromosome 19.[erratum appears in EMBO J 1992 Mar;11(3):1228]. EMBO Journal **10**:3941–3950.

36. **Saudan, P., J. Vlach, and P. Beard.** 2000. Inhibition of S-phase progression by adeno-associated virus Rep78 protein is mediated by hypophosphorylated pRb. EMBO Journal **19**:4351–4361.

37. **Schwartz, R. A., C. T. Carson, C. Schuberth, and M. D. Weitzman.** 2009. Adeno-associated virus replication induces a DNA damage response coordinated by DNA-dependent protein kinase. J Virol **83**:6269–6278.

38. **Snyder, R. O., D. S. Im, and N. Muzyczka.** 1990. Evidence for covalent attachment of the adeno-associated virus (AAV) rep protein to the ends of the AAV genome. Journal of Virology **64**:6204–6213.

39. **Timpe, J. M., K. C. Verrill, and J. P. Trempe.** 2006. Effects of adeno-associated virus on adenovirus replication and gene expression during coinfection. Journal of Virology **80**:7807–7815.

40. **Untergasser, A., I. Cutcutache, T. Koressaar, J. Ye, B. C. Faircloth, M. Remm, and S. G. Rozen.** 2012. Primer3--new capabilities and interfaces. Nucleic Acids Res. **40**:e115.

41. **Walz, C., A. Deprez, T. Dupressoir, M. Dürst, M. Rabreau, and J. R. Schlehofer.** 1997. Interaction of human papillomavirus type 16 and adeno-associated virus type 2 co-infecting human cervical epithelium. J. Gen. Virol. **78 (Pt 6)**:1441–1452.

42. **Ward, P., M. Falkenberg, P. Elias, M. Weitzman, and R. M. Linden.** 2001. Rep-dependent initiation of adeno-associated virus type 2 DNA replication by a herpes simplex

virus type 1 replication complex in a reconstituted system. Journal of Virology **75**:10250–10258.

43. **Weindler, F. W., and R. Heilbronn.** 1991. A subset of herpes simplex virus replication genes provides helper functions for productive adeno-associated virus replication. Journal of Virology **65**:2476–2483.
44. **Weitzman, M. D., K. J. Fisher, and J. M. Wilson.** 1996. Recruitment of wild-type and recombinant adeno-associated virus into adenovirus replication centers. Journal of Virology **70**:1845–1854.
45. **Zarate-Perez, F., J. Mansilla-Soto, M. Bardelli, J. W. Burgner, M. Villamil-Jarauta, D. Kekilli, M. Samso, R. M. Linden, and C. R. Escalante.** 2012. Oligomeric Properties of Adeno-Associated Virus Rep68 Reflect Its Multifunctionality. Journal of Virology **87**:1232–1241.
46. **Zarate-Perez, F., J. Mansilla-Soto, M. Bardelli, J. W. Burgner, M. Villamil-Jarauta, D. Kekilli, M. Samso, R. M. Linden, and C. R. Escalante.** 2013. Oligomeric properties of adeno-associated virus Rep68 reflect its multifunctionality. J. Virol. **87**:1232–1241.
47. **Zeman, M. K., and K. A. Cimprich.** 2013. Causes and consequences of replication stress. Nat Cell Biol **16**:2–9.
48. **Zeng, Y., K. C. Forbes, Z. Wu, S. Moreno, H. Piwnica-Worms, and T. Enoch.** 1998. Replication checkpoint requires phosphorylation of the phosphatase Cdc25 by Cds1 or Chk1. Nature **395**:507–510.
49. **Zou, L., and S. J. Elledge.** 2003. Sensing DNA damage through ATRIP recognition of RPA-ssDNA complexes. Science **300**:1542–1548.

7. Phenotypic Characterization of the Novel Mutant AAV2 Rep78-D371Y Protein

Original title

Phenotypic Profiling of the Mutant AAV2 Rep78-D371Y Protein Reveals its Potential for the Design of Novel HSV/AAV-Hybrid Gene Transfer Vectors

Own Contribution

- Cloning of plasmids
- Western Blots
- Southern Blots
- Replication assays
- Packaging assays
- Immuno-fluorescence
- Confocal microscopy
- Writing the manuscript
- Figure preparations

7.1. Background and Rationale

In the past decade, HSV/AAV hybrid gene therapy vectors have been generated and used successfully to deliver a variety of transgenes to target cells *in vitro* and *in vivo* (1, 4, 5, 9, 13). Despite this success, HSV/AAV hybrid vectors still are far away from being a reliable and robust therapeutic gene therapy tools in human medicine. Two main factors reduce the performance of HSV/AAV hybrid vectors: (i) the inevitable expression of the toxic *rep* genes is needed to establish site-specific integration of the transgene and (ii) the Rep-mediated inhibition of HSV-1 DNA replication, especially during vector production. The first factor is one of the main reasons for safety concerns since the AAV2 Rep proteins induce a cellular DDR and apoptosis (2, 7, 14, 15). The inhibition of HSV-1 replication on the other hand results in poor hybrid vector titers (9). To overcome these drawbacks HSV/AAV hybrid vectors were constructed which express little or even no Rep during packaging. However, in transduced cells providing the Cre recombinase, Rep was expressed upon site-specific recombination to establish site-specific integration of the transgene DNA (12). Since this strategy relies on the expression of the Cre recombinase in the target cells, the therapeutic applicability is limited. Therefore, the need for new strategies to overcome the drawbacks of HSV/AAV hybrid vectors is still amenable.

Here, we describe the phenotype of a newly emerged Rep-helicase mutant (D371Y), which has the potential to overcome the aforementioned drawbacks of HSV/AAV hybrid vectors expressing the *rep* ORF during packaging and after transduction. In particular, we demonstrate that Rep78D371Y supports the simultaneous replication of both AAV2 and HSV-1 DNA replication. Moreover we found that overall Rep toxicity was drastically reduced in cells expressing the new mutant Rep78D371Y. These findings indicate that Rep78D371Y can potentially improve the performance of HSV/AAV hybrid vectors.

7.2. Findings

Expression levels of the wild-type and mutant *rep* genes. In order to characterize the phenotype of the Rep-helicase mutant D371Y, which harbors a Tyrosine (Y) instead of an Aspartic acid (D) at position 371, we compared three Rep constructs; (i) the wt Rep78 consisting of the full-length Rep ORF, (ii) the Rep78 ORF with the helicase-null mutant K340H, which harbors a mutation within the Walker A motif of the ATPase helicase domain

(a Histidine instead of a Lysin at position 340) (3, 11) and (iii) the aforementioned Rep78 helicase mutant D371Y (Fig. 7.1 A). All *rep* genes are under the control of the constitutively active CMV IE1 enhancer/promoter and cloned into the expression vector pcDNA3.1+. To estimate the expression levels, Vero cells were transfected with the corresponding plasmids and subjected to Western analysis 24 hrs post transfection using a Rep-specific antibody (Fig. 7.1 B). Actin staining served as a loading control. All three Rep constructs expressed the *rep* genes at comparable levels. The Rep78 and the Rep52 variants are indicated.

Both Rep-helicase mutants Rep78D371Y and Rep78K340H allow HSV-1 DNA replication, whereas wt Rep78 does not. Effects of the different Rep constructs on HSV-1 DNA replication were assessed using the HSV-1 TetO-TetR DNA visualization system (6). In brief, Vero cells were transfected with the plasmid pHSV-TetO, which harbors 35 TetR-binding sites together with the different Rep expressing constructs. One day post transfection, the cells were superinfected with wt HSV-1 (MOI 2) to provide all replication factors necessary to replicate the pHSV-TetO replicon. The expression of a TetR-EYFP fusion protein allows the visualization of the pHSV-TetO replication compartments (RCs). The Rep-helicase mutants Rep78K340H and Rep78D371Y both allowed the formation of mature pHSV-TetO RCs, whereas the wt Rep78 did not (Fig. 7.2 A). In addition, a pHSVGFP packaging assay was utilized to quantify the effects of the different Rep constructs. Vero 2-2 cells were transfected with the HSV-1 amplicon plasmid pHSVGFP together with a HSV-1 helper DNA (fHSV BAC) providing HSV-1 replication factors and the Rep expressing constructs. Three days post transfection the pHSVGFP amplicons were harvested and titrated on Vero cells. Compared to the negative control, the wt Rep78 proteins reduced the pHSVGFP vector titers by 100-fold, while the effects of both Rep-helicase mutants were less pronounced (less than 10-fold) and at an equal level (Fig. 7.2 B). The inability of the helicase-null mutant Rep78K340H to inhibit HSV-1 DNA replication was expected and was described before (7). This led to the assumption that similar to Rep78K340H, the mutant Rep78D371Y may not provide helicase activity either and therefore may not be compatible with AAV2 DNA replication.

Unlike Rep78K340H, the mutant Rep78D371Y supports AAV2 DNA replication. To test the capability of the Rep constructs to support AAV2 DNA replication we utilized the pAV2-LacO DNA replication system. Vero cells were transfected with the AAV2 replicon pAV2-LacO together with the different Rep expressing constructs and a plasmid expressing the LacI-EYFP fusion protein. One day post transfection, the cells were superinfected with wt HSV-1. Another day later the cells were fixed, stained with a Rep-specific antibody and the

formation of pAV2-LacO RCs was assessed. Surprisingly, unlike Rep78K340H, Rep78D371Y fully supported the formation of pAV2-LacO RCs (Fig. 7.3 A). To confirm the observed support for AAV2 DNA replication by Rep78D381Y, we utilized an AAV2 DNA replication assay based on Southern analysis. Vero cells were transfected with the AAV2 replicon plasmid pAV2GFP together with the different Rep constructs and one day later superinfected with HSV-1 to provide helper factors for AAV2 replication. Three days post infection extra-chromosomal DNA was extracted, digested with *Dpn*I to cut bacterial input DNA and subjected to Southern analysis using a GFP-specific probe (Fig. 7.3 B). Rescued monomeric (ITRm) and dimeric (ITRd) pAV2GFP replication intermediates (*Dpn*I resistant) are indicated. As expected, the helicase null mutant Rep78K340H did not support the formation of any pAV2GFP intermediates, whereas the wt Rep78 and the mutant Rep78D371Y both did.

Notably, the formation of HSV-1 RCs is dramatically reduced when either AAV2 is replicating, or the wt Rep proteins are present in the same cell (7, 8).These findings however suggest that the Rep78D371Y mutant may allow the simultaneous replication of HSV-1 and AAV2 DNA in the same cell. To test this hypothesis, we utilized the systems to visualize the formation of HSV-1 and AAV2 RCs simultaneously by co-transfecting Vero cells with the corresponding replicon plasmids pHSV-TetO and pAV2-LacO together with the different Rep constructs. As expected, the formation of AAV2 RCs was observed with the wt Rep78 but not with the mutant Rep78K340H, whereas the formation of HSV-1 RCs was observed with the mutant Rep78K340H, but not the wt Rep78. However, the mutant Rep78D371Y indeed allowed the simultaneous and comparable formation of RCs from both viruses AAV2 and HSV-1 (Fig. 7.4).

The mutant Rep78D371Y is less toxic than the wt Rep78. In previous studies, we and others reported that the major AAV2 Rep68 and Rep78 proteins induce a DNA-damage response (DDR) in transfected cells characterized by the activation (i.e. phosphorylation) of the replication protein A (pRPA), the sensor-kinase ataxia-telangiectasia mutated (pATM) and the histone γH2AX (2, 7, 14, 15). We also demonstrated that the combined Rep DNA-binding- and ATPase/helicase domain are necessary to induce the cellular DDR (7). Therefore we addressed the question whether the mutant Rep78D371Y is also able to induce a DDR in transfected cells. Vero cells expressing the different Rep constructs were fixed at 48 h after transfection and stained with antibodies specific for either pATM (S1981), pRPA32 (S4/S8) or γH2AX (S139). Rep expression was detected with Rep-specific antibodies. Rep- and GFP-positive cells were counted and scored for the staining of the

different DDR markers (Fig. 7.5 A-C). Interestingly, the DDR response is reduced 20-60% in cells expressing the Rep78D371Y protein when compared with the wt Rep78 expressing cells. As shown in a previous study, the DDR induced by Rep78K340H was reduced to basic levels when compared with the negative control (pcDNA-EGFP) (7). In a next step, we examined the capability of Rep78D371Y to induce apoptosis in transfected cells, since the Rep-domains responsible of inducing a DDR coincide with the domains responsible for inducing apoptosis (7). To do so, Vero cells were transfected with the different Rep constructs along with a GFP expressing plasmid to identify successfully transfected cells. Three days later the cells were stained with Cy5-conjugated annexin V and analyzed by screening of GFP-positive cells (flow cytometry) for the induction of apoptosis (Fig. 7.5 D). Similar to the mutant Rep78K340H proteins, the mutant Rep78D371Y proteins did not induce apoptosis, whereas the wt Rep78 proteins did. These results indicate that the overall toxicity of the mutant Rep78D371Y is clearly reduced compared to the wt Rep78.

The titers of HSV/AAV hybrid vectors harboring the mutant Rep78D371Y are 10-fold higher than those of hybrid vectors harboring the wt Rep78. HSV/AAV hybrid vectors have previously been generated to combine the advantages and to overcome the drawbacks of HSV-1 and AAV2 vectors (9, 10, 16). However, due to Rep-mediated toxicity and inhibiton of HSV-1 DNA replication, HSV/AAV hybrid vectors harboring the wt *rep* ORF were not promising because vector titers were too low (9). Since the newly characterized mutant Rep78D371Y proteins allow the simultaneous replication of both AAV2 and HSV-1 and in addition the overall toxicity is reduced, we included the mutation D371Y into the pre-existing HSV/AAV hybrid vectors, replacing the wt Rep78 ORF. Specifically, we cloned the complete *rep* ORF containing the mutation D371Y into the HSV/AAV hybrid vector backbone pHyGFPa resulting in the mutant hybrid vector pHyD371YGFPa. This hybrid vector next was packaged in Vero 2-2 cells as described above. Three days post transfection the hybrid vectors were harvested and titrated on Vero cells. The HSV/AAV hybrid vector harboring the wt *rep* ORF (pHyRaNGFPa) (9) and the HSV-1 amplicon vector harboring no AAV2 elements (pHSVGFP) were used as controls. As predicted, the titers of the HSV/AAV hybrid vectors encoding the Rep D371Y mutation (pHyD371YGFPa) were up to 10-fold higher than those of hybrid vectors encoding the wt Rep (pHyRaNGFPa) (Fig. 7.6). However, the mutant hybrid vector titers were still approximately 10-fold lower than those of the standard HSV-1 amplicon vectors.

7.3. Conclusions

In this study, we elaborated the phenotypic profile of the novel AAV2 Rep helicase mutant Rep78D371Y. We assessed whether Rep78D371Y proteins are capable of promoting AAV2 DNA replication. We found that unlike the helicase-null mutant Rep78K340H proteins, the Rep78D371Y proteins are fully compatible with AAV2 DNA replication. We also tested the capability of Rep78D371Y proteins to inhibit HSV-1 DNA replication. Interestingly, our results revealed that like the helicase-null mutant Rep78K340H, Rep78D371Y also lost the capability to inhibit HSV-1 DNA replication. In a next step, we assessed the induction of a DDR in Rep transfected cells. Specifically, we tested whether the mutant Rep78D371Y proteins induce the activation of the following DDR marker proteins: (i) the sensor-kinase ataxia-telangiectasia mutated (pATM), (ii) the replication protein A (pRPA) and (iii) the histone H2AX (γH2AX). Surprisingly, the levels of activated DDR proteins in Rep78D371Y expressing cells were 20-60% lower than in cells expressing wt Rep78. Moreover, we found that the Rep78D371Y proteins unlike their wt Rep78 counterparts do not induce apoptosis in cells. These findings suggest that overall toxicity of Rep78 is significantly reduced in cells expressing the mutant Rep78D371Y. The AAV2 mutant and wt Rep78 activities are summarized in Table 7.1.

The phenotypic profile of Rep78D371Y had a beneficial impact on the production of next generation HSV/AAV hybrid gene therapy vectors. Due to the inability of Rep78D371Y to inhibit HSV-1 DNA replication, HSV/AAV hybrid vector titers were elevated up to 10-fold when expressing the mutant Rep78D371Y instead of the wt Rep78. In addition, the reduced Rep-mediated cell toxicity of Rep78D371Y proteins may improve the overall safety profile of the vector system.

7.4. Materials and Methods

Cells and viruses

Vero and Vero 2-2 cells were maintained, wt HSV-1 (F-strain) was produced and titrated, and cells were infected as described in section 5 of this thesis. Transfections were performed using the Lipofectamine™ LTX and Plus™ reagents from Invitrogen™ according to the manufacturers' protocol.

Plasmids

The plasmids expressing the wt Rep78 (pcDNA.Rep78) and the mutant Rep78K340H (pcDNA.Rep78K340H) genes were described in section 5 of this thesis. The plasmid expressing the mutant Rep78D371Y (pcDNA.Rep78D371Y) was cloned by PCR as follows: A first round of PCR was performed using the pcDNA.Rep78 plasmid DNA as a template with primers reaching from the first *Bam*HI-site (nt1040) to the *Acc*I-site (nt1420) where in the reverse primer the G→T substitution at position 1431 was introduced (for. primer: GCAGTGGATCCAGGAGGACCAGGCCTCATA / rev. primer: ACCAGATCACCATCTTGTA GACACAGTCGT). A second round of PCR was performed with primers reaching from *Acc*I-site (nt1413) to the *Xho*I-site (nt2225) (for. primer: CCCTTCAACGACTGTGTCT ACAAGATGGTG / rev. primer: CTTCAGAGAGAGTGTCC TCGAGCCAATCTG). The two PCR fragments were ligated between the *Bam*HI and *Xho*I sites of pcDNA.Rep78. The empty plasmid backbone pcDNA3.1+ was purchased from Invitrogen™ (Cat.no. V790-20). The HSV/AAV hybrid vector plasmid pHyRaNGFPa was described elsewhere (9). In brief, the plasmid pRep harboring the full-length Rep ORF was cut with *Not*I and the overhang-ends were blunt-end repaired with T4 DNA polymerase. This 2.5-kb fragment was inserted into the blunt-ended *Sph*I site of pHSVNot resulting in pHyRa. In parallel, the *Bgl*II fragment was excised from the pAV2GFP plasmid which contains the ITR-flanked GFP cassette and was inserted into the *BamH*I site of pUC18-Not. The resulting plasmid pAV2GFP-Not was cleaved with *Not*I and the 2.3-kb fragment containing the ITR-flanked transgene *egfp* was inserted into the unique *Not*I site on pHyRa, forming pHyRaNGFPa. To generate the plasmid pHyRD371YGFPa, the same strategy was used as to generate pHyRaNGFPa, but instead a pRep plasmid was used harboring the mutation D371Y (pRepD371Y). pRepD371Y was cloned by ligating the PstI fragment from the pcDNA.Rep78D371Y template directly into the PstI cleaved target plasmid pRep.

Western Blotting

Western blotting was performed as described in section 5 of this thesis, except that the following expression plasmids were used: (i) pcDNA.Rep78, (ii) pcDNA.Rep78K340H, (iii) pcDNA.Rep78D371Y and (iv) the empty backbone plasmid pcDNA3.1+.

Immunofluorescence analysis

Visualization of AAV2 replication compartments and IF-staining of the AAV2 Rep proteins were performed as described in section 5 of this thesis and in reference (6). Visualization of

HSV-1 RCs was performed as described in section 6 of this thesis and in reference (8). In addition 0.5µg of the different Rep expressing plasmids were co-transfected with the corresponding HSV-1 or AAV2 visualization amplicons. Screening of cells expressing the different Rep expressing constructs pcDNA.Rep78, pcDNA.Rep78K340H or pcDNA.Rep78D371Y for the DDR markers pATM, pRPA and γH2AX was described before in section 5 of this thesis.

HSV-1 amplicon and HSV/AAV hybrid vector packaging assay

Packaging and titration of pHSVGFP amplicons was described in section 5 of this thesis. The different Rep expressing plasmids pcDNA.Rep78, pcDNA.Rep78K340H, or pcDNA.Rep78D371Y were co-transfected with the HSV-1 amplicon vector pHSVGFP or the HSV/AAV hybrid vectors pHyRaNGFPa or pHyRD371YGFPa together with the HSV-1 helper genome (fHSVΔpacΔ27). Harvesting and titration of vector stocks was performed as described in section 5 of this thesis.

Southern Blotting

The different Rep expressing plasmids pcDNA.Rep78, pcDNA.Rep78K340H, pcDNA.Rep78D371Y or the empty backbone plasmid pcDNA3.1+ were co-transfected together with the AAV2 replicon pAV2GFP and superinfected with wtHSV-1 (MOI 2) one day later. AAV2 replication intermediates were visualized by Southern analysis as described in section 5 of this thsis.

Flow cytometry analysis

Apoptotic cells expressing the different Rep constructs pcDNA.Rep78, pcDNA.Rep78K340H, pcDNA.Rep78D371Y and the empty backbone plasmid pcDNA3.1+ were stained and counted by flow cytometry as described in section 5 of this thesis.

7.5. Figure Legends & Figures

Figure 7.1. The AAV2 Rep expression plasmids. (A) Schematic representation of the AAV2 Rep proteins analyzed in this study; (i) the wild-type (wt) Rep78, (ii) the helicase-deficient mutant Rep78K340H and (iii) the helicase mutant Rep78D371Y. The ATPase/helicase domain (dark grey box) and the mutations (black lines) are indicated. A detailed scheme of the helicase domain (aa 225-490) is shown below. The mutation K340H is located within the Walker motif A whereas the mutation D371Y is located between the Walker motifs A and B. The exact locations within the Rep aa-sequence are indicated on the bottom of the scheme. **(B)** Rep expression levels. Plasmids encoding either the wt Rep78, the mutat Rep78K340H, the mutant Rep78D371Y or the empty vector plasmid pcDNA were transfected into Vero cells. One day post transfection, the cells were harvested and processed for Western analysis using a Rep-specific antibody. The Rep78- and the Rep52 variants are indicated on the right. Detection of actin served as a loading control.

Figure 7.2. Evaluation of the different Rep constructs for their ability to promote HSV-1 replication. (A) Replication of an HSV-1 replicon is visualized with plasmid pHSV-TetO consisting of the HSV-1 oriS and a tetO-cassette containing 35x tetR binding sites. In presence of HSV-1 replication factors, the accumulation of pHSV-TetO replication products is visualized by binding of an EYFP-TetR fusion protein. Cells expressing the different Rep constructs (indicated on top) were analyzed for the ability to inhibit HSV-1 replication. The Rep proteins were stained with a Rep-specific antibody (red insets). Size bar; 5µm. **(B)** Effects of the Rep constructs on HSV-1 amplicon vector packaging. Vero 2-2 cells were transfected with the HSV-1 amplicon DNA pHSVGFP, packaging-defective HSV-1 helper DNA fHSVΔpacΔ27Δkn, HSV-1 ICP27 encoding plasmid pEBHICP27 and different Rep expression plasmids. 72 hrs post transfection the pHSVGFP amplicon vector particles were harvested and titrated on Vero cells. The data is shown as means ± SD from three independent experiments. Asterisks indicate statistically significant differences based on a paired two-tail students t-test (** = $p<0.01$).

Figure 7.3. Effects of the different Rep constructs on AAV2 DNA replication. (A) Replication of an AAV2 replicon is visualized with plasmid pAV2-LacO, which is harbouring a LacO-cassette consisting of 40 LacI binding sites flanked by AAV2 ITRs. In presence of the different Rep constructs and HSV-1 helper factors, the accumulation of pAV2-LacO replication products is visualized by binding of an EYFP-LacI fusion protein. The Rep proteins were stained with a Rep-specific antibody (red insets). Size bar; 5µm. **(B)** Vero 2-2

cells were transfected with pAV2GFP and the different Rep constructs as indicated. One day post transfection, the cells were super-infected with HSV-1 (MOI 2) and 48hrs later subjected to HIRT DNA extraction. The extracted extrachromosomal DNA was digested with DpnI and analyzed by Southern blotting with a DIG-labeled probe specific for EGFP. The rescued monomeric (ITRm) and dimeric (ITRd) replication intermediates are indicated on the right. As a positive control, 0.1 ng of the excised (Bgl II digested) ITR cassette from pAV2GFP was loaded.

Figure 7.4. Co-visualization of competing AAV2 and HSV-1 replication in the same cell. DNA Replication of AAV2 and HSV-1 was visualized in the same cell by transfecting Vero 2-2 cells with the replicons pAV2-LacO and pHSV-TetO, respectively, together with the Rep constructs as indicated on the top. One day later, the cells were super-infeced with HSV-1 (MOI 2). Replication products from pAV2-LacO were visualized with the fusion protein mRFP-LacI and replication products from pHSV-TetR were visualized with the fusion protein EYFP-TetR. Size bar; 2.5µm.

Figure 7.5. Effects of the different Rep constructs on the cell. (A-C) Screening of the Rep constructs for their ability to induce a DNA-damage response. Vero cells were transfected with plasmids expressing either wtRep78, Rep78K340H or Rep78D371Y. The empty vector plasmid pcDNA-EGFP served as a negative control. After two days, the cells were fixed and stained with antibodies specific for Rep and either pRPA (S3/4), pATM (S1981) or γH2AX (S139). Rep^+- or GFP^+-cells were scored for staining of the DDR markers. **(D)** Screening of the Rep constructs for their ability to induce apoptosis in the transfected cell. Vero cells were transfected with plasmids as described in panels A-C together with an EGFP expressing plasmid. Three days later, the cells were stained with Cy5-conjugated annexin V and analyzed by flow cytometry with filters specific for EGFP (transfected cells) and Cy5 (apoptotic cells). The data is shown as means ± SD from three independent experiments.

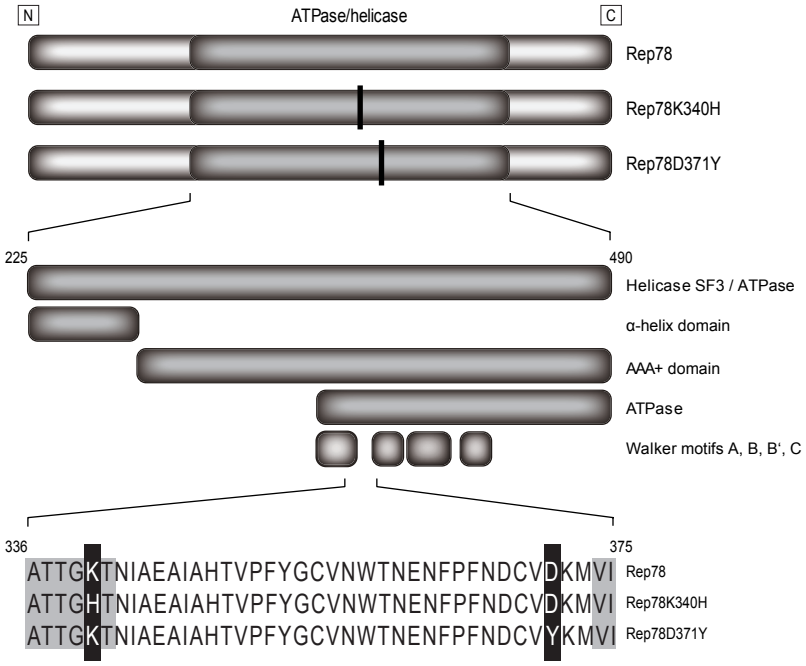
Figure 7.6. HSV/AAV hybrid vector replication and packaging. VERO 2-2 cells were transfected with the following vector plasmid DNAs: the HSV/AAV hybrid vector pHyRaNGFPa containing the wtRep ORF or the HSV/AAV hybrid vector pHyRD371YGFPa containing the D371Y helicase mutant Rep ORF. Packaging of the vectors into HSV-1 virions was facilitated by co-transfecting a packaging-defective HSV-1 helper DNA fHSVΔpacΔ27Δkn and the HSV-1 ICP27 encoding plasmid pEBHICP27. Vector particles were harvested 3 days after transfection and titrated on Vero cells. Packaging of the HSV-1

vector plasmid pHSVGFP served as positive control. Mock infected Vero cells served as a negative control for the titration. The data is shown as means ± SD from three independent experiments. Asterisks indicate statistically significant differences based on a paired two-tail students t-test (*= $p<0.05$).

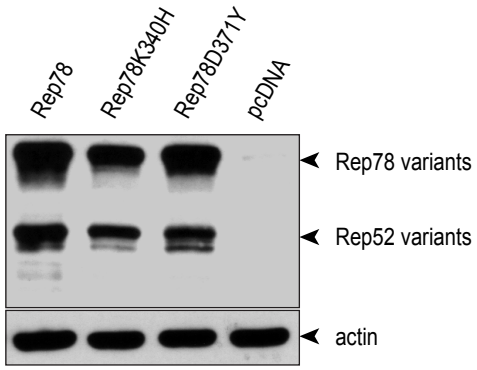
Table 7.1. Summary of AAV2 Rep activities. The activities of the wt Rep78, the mutants Rep78K340H and Rep78D371Y were scored for their ability to (i) promote AAV2 replication, (ii) inhibit HSV-1 replication, (iii) induce a DDR and (iv) induce apoptosis; (-) no score, (+) minimal score, (++) intermediate score, (+++) high score.

A

Fig. 7.1

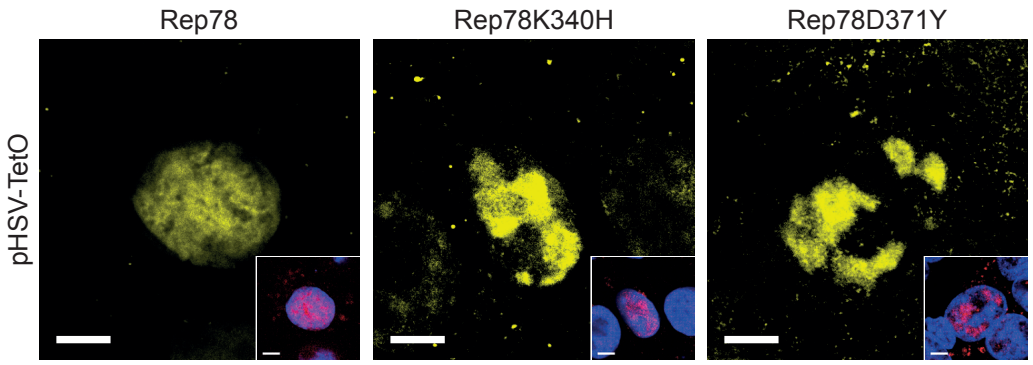

N
ATPase/helicase
C
Rep78
Rep78K340H
Rep78D371Y
225
490
Helicase SF3 / ATPase
α-helix domain
AAA+ domain
ATPase
Walker motifs A, B, B', C
336
375
ATTGKTNIAEAIAHTVPFYGCVNWTNENFPFNDCVDKMVI Rep78
ATTGHTNIAEAIAHTVPFYGCVNWTNENFPFNDCVDKMVI Rep78K340H
ATTGKTNIAEAIAHTVPFYGCVNWTNENFPFNDCVYKMVI Rep78D371Y

B

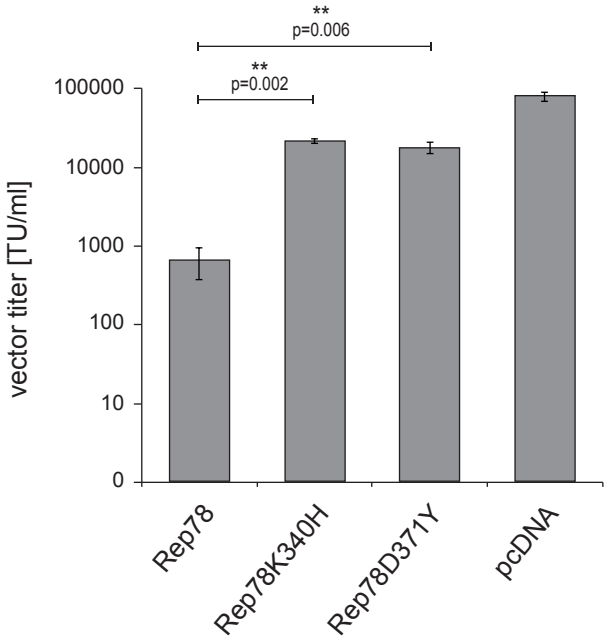

Rep78
Rep78K340H
Rep78D371Y
pcDNA
Rep78 variants
Rep52 variants
actin

A

Fig. 7.2

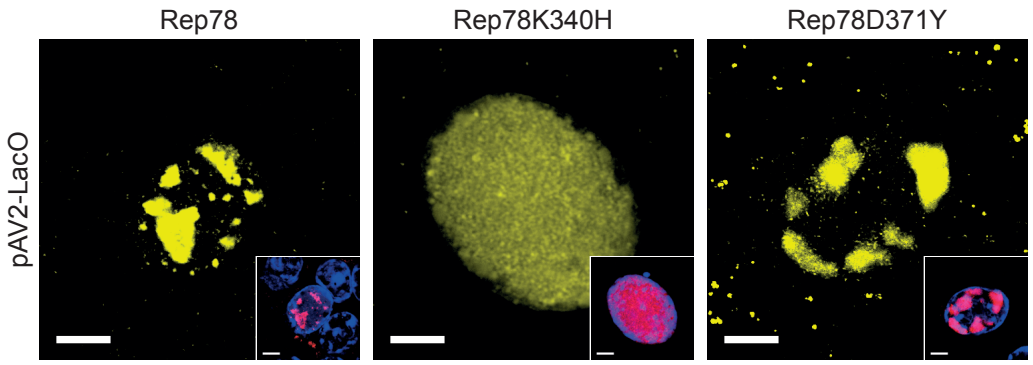

Rep78
Rep78K340H
Rep78D371Y
pHSV-TetO

B

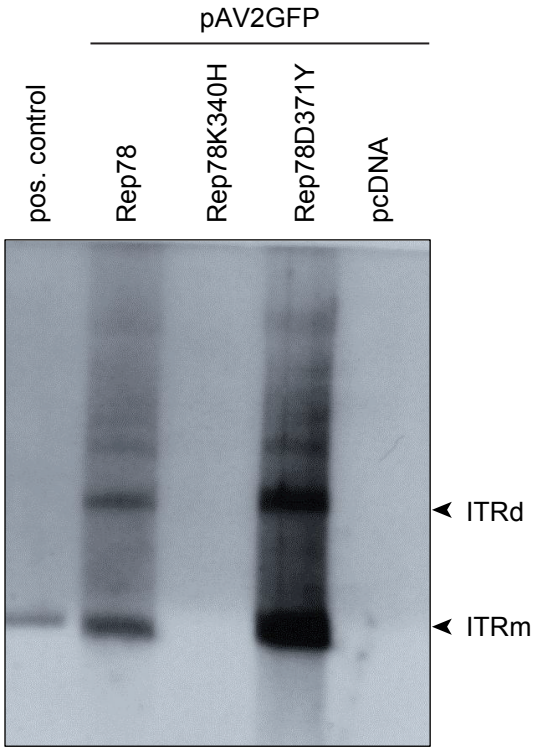

**
p=0.006
**
p=0.002
100000
10000
1000
100
10
0
vector titer [TU/ml]
Rep78
Rep78K340H
Rep78D371Y
pcDNA

A

Fig. 7.3


Rep78
Rep78K340H
Rep78D371Y
pAV2-LacO

B


pAV2GFP
pos. control
Rep78
Rep78K340H
Rep78D371Y
pcDNA
ITRd
ITRm

A

Fig. 7.4

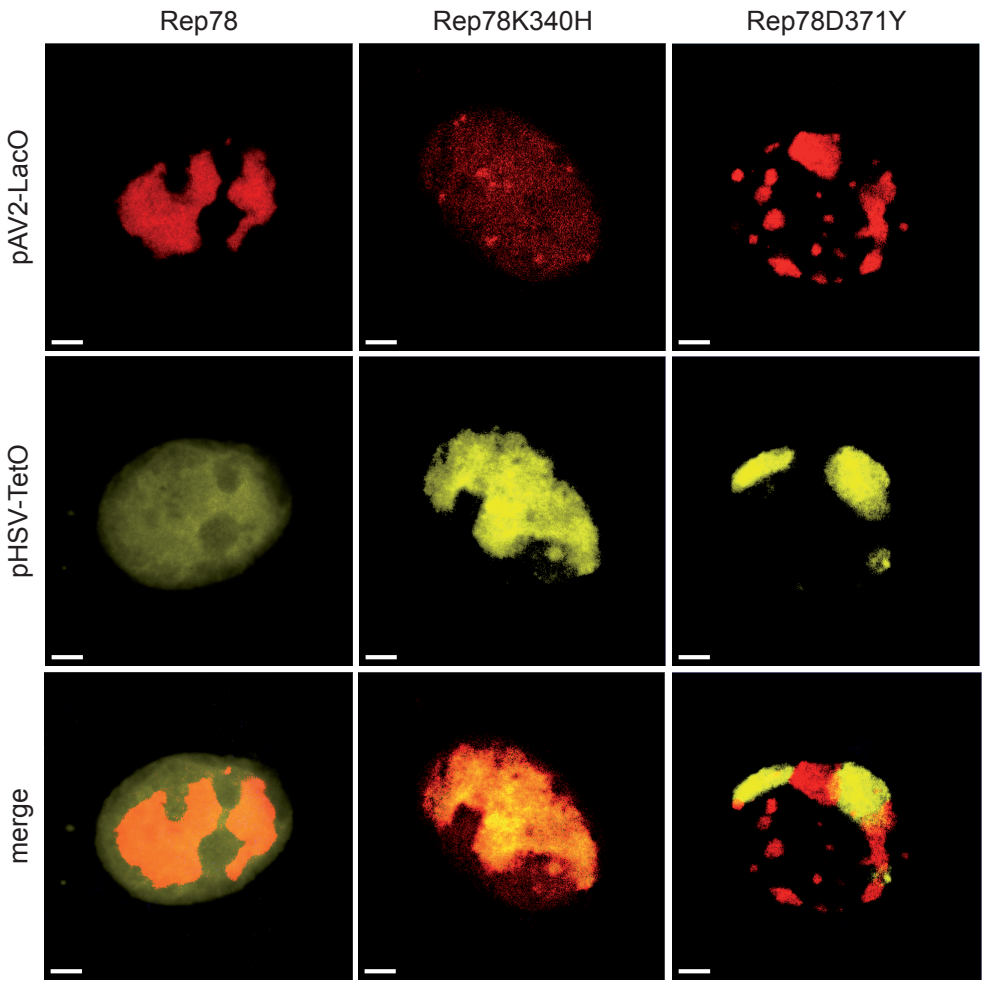
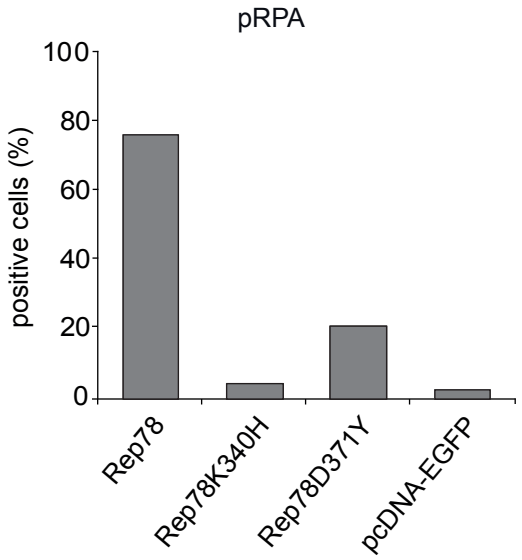
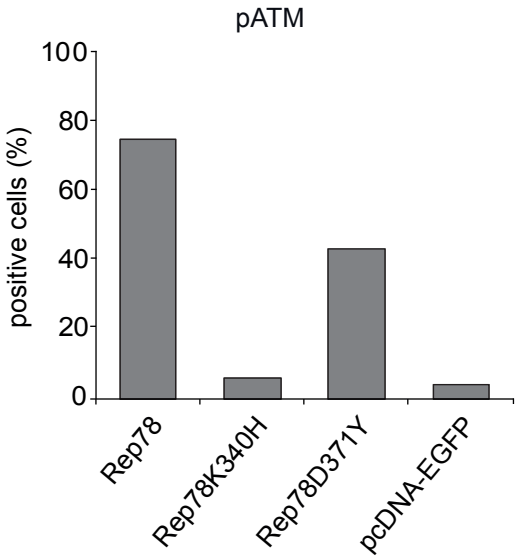


Fig. 7.5

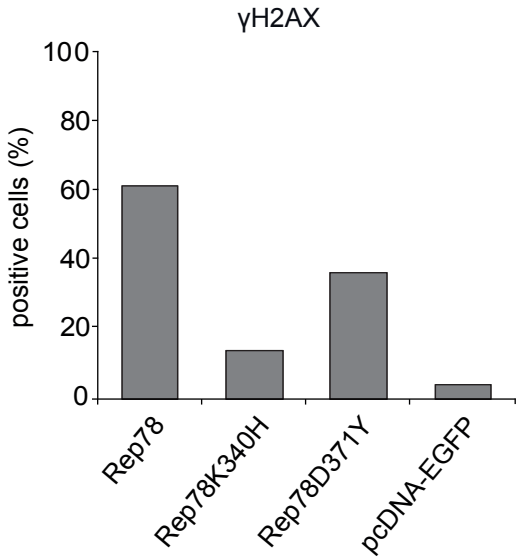
A


pRPA
positive cells (%)
100
80
60
40
20
0
Rep78
Rep78K340H
Rep78D371Y
pcDNA-EGFP

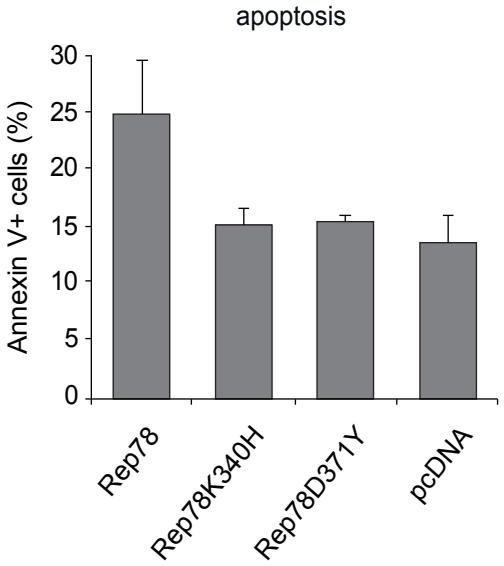
B


pATM
positive cells (%)
100
80
60
40
20
0
Rep78
Rep78K340H
Rep78D371Y
pcDNA-EGFP

C


γH2AX
positive cells (%)
100
80
60
40
20
0
Rep78
Rep78K340H
Rep78D371Y
pcDNA-EGFP

D


apoptosis
Annexin V+ cells (%)
30
25
20
15
10
5
0
Rep78
Rep78K340H
Rep78D371Y
pcDNA

A

Fig. 7.6

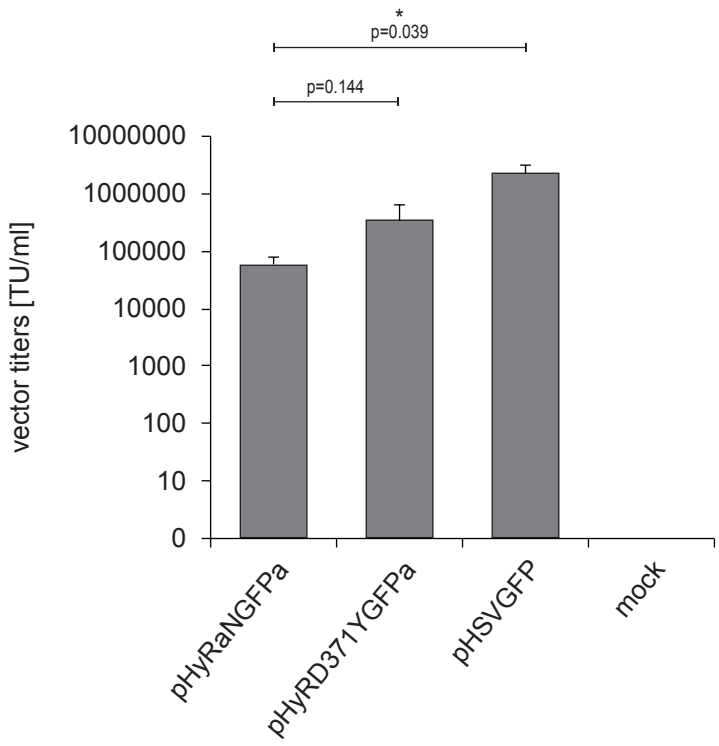

*
p=0.039
p=0.144
10000000
1000000
100000
10000
1000
100
10
0
vector titers [TU/ml]
pHyRaNGFPa
pHyRD371YGFPa
pHSVGFP
mock

Table 7.1

Table 1. Summary of AAV2 Rep activities

Rep	AAV2 replication	HSV-1 replication	DNA damage response	apoptosis
78	+++	-	+++	+++
78K340H	-	++	-	-
78D371Y	+++	++	+	-

7.6. References (Chapter 7)

1. **Bakowska, J. C., M. V. Di Maria, S. M. Camp, Y. Wang, P. D. Allen, and X. O. Breakefield.** 2003. Targeted transgene integration into transgenic mouse fibroblasts carrying the full-length human AAVS1 locus mediated by HSV/AAV rep(+) hybrid amplicon vector. Gene Ther. **10**:1691–1702. doi:10.1038/sj.gt.3302061.
2. **Berthet, C., K. Raj, P. Saudan, and P. Beard.** 2005. How adeno-associated virus Rep78 protein arrests cells completely in S phase. Proceedings of the National Academy of Sciences of the United States of America **102**:13634–13639.
3. **Chejanovsky, N., and B. J. Carter.** 1990. Mutation of a consensus purine nucleotide binding site in the adeno-associated virus rep gene generates a dominant negative phenotype for DNA replication. J. Virol. **64**:1764–1770.
4. **Cortés, M. L., A. Oehmig, O. Saydam, J. D. Sanford, K. F. Perry, C. Fraefel, and X. O. Breakefield.** 2008. Targeted integration of functional human ATM cDNA into genome mediated by HSV/AAV hybrid amplicon vector. Mol. Ther. **16**:81–88. doi:10.1038/sj.mt.6300338.
5. **Fraefel, C., D. R. Jacoby, C. Lage, H. Hilderbrand, J. Y. Chou, F. W. Alt, X. O. Breakefield, and J. A. Majzoub.** 1997. Gene transfer into hepatocytes mediated by helper virus-free HSV/AAV hybrid vectors. Molecular Medicine **3**:813–825.
6. **Glauser, D. L., O. Saydam, N. A. Balsiger, I. Heid, R. M. Linden, M. Ackermann, and C. Fraefel.** 2005. Four-dimensional visualization of the simultaneous activity of alternative adeno-associated virus replication origins. Journal of Virology **79**:12218–12230.
7. **Glauser, D. L., M. Seyffert, R. Strasser, M. Franchini, A. S. Laimbacher, C. Dresch, A. P. de Oliveira, R. Vogel, H. Büning, A. Salvetti, M. Ackermann, and C. Fraefel.** 2010. Inhibition of herpes simplex virus type 1 replication by adeno-associated virus rep proteins depends on their combined DNA-binding and ATPase/helicase activities. J. Virol. **84**:3808–3824. doi:10.1128/JVI.01503-09.
8. **Glauser, D. L., R. Strasser, A. S. Laimbacher, O. Saydam, N. Clément, R. M. Linden, M. Ackermann, and C. Fraefel.** 2007. Live covisualization of competing adeno-associated virus and herpes simplex virus type 1 DNA replication: molecular mechanisms of interaction. Journal of Virology **81**:4732–4743.
9. **Heister, T., I. Heid, M. Ackermann, and C. Fraefel.** 2002. Herpes simplex virus type 1/adeno-associated virus hybrid vectors mediate site-specific integration at the adeno-

associated virus preintegration site, AAVS1, on human chromosome 19. Journal of Virology **76**:7163–7173.

10. **Johnston, K. M., D. Jacoby, P. A. Pechan, C. Fraefel, P. Borghesani, D. Schuback, R. J. Dunn, F. I. Smith, and X. O. Breakefield.** 1997. HSV/AAV hybrid amplicon vectors extend transgene expression in human glioma cells. Human Gene Therapy **8**:359–370.
11. **Kyostio, S. R., R. S. Wonderling, and R. A. Owens.** 1995. Negative regulation of the adeno-associated virus (AAV) P5 promoter involves both the P5 rep binding site and the consensus ATP-binding motif of the AAV Rep68 protein. Journal of Virology **69**:6787–6796.
12. **Liu, Q., C. F. Perez, and Y. Wang.** 2006. Efficient site-specific integration of large transgenes by an enhanced herpes simplex virus/adeno-associated virus hybrid amplicon vector. Journal of Virology **80**:1672–1679.
13. **Oehmig, A., M. L. Cortes, K. F. Perry, M. Sena-Esteves, C. Fraefel, and X. O. Breakefield.** 2007. Integration of active human beta-galactosidase gene (100 kb) into genome using HSV/AAV amplicon vector. Gene Ther **14**:1078–1091. "http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=17460718 ".
14. **Saudan, P., J. Vlach, and P. Beard.** 2000. Inhibition of S-phase progression by adeno-associated virus Rep78 protein is mediated by hypophosphorylated pRb. EMBO Journal **19**:4351–4361.
15. **Schwartz, R. A., C. T. Carson, C. Schuberth, and M. D. Weitzman.** 2009. Adeno-associated virus replication induces a DNA damage response coordinated by DNA-dependent protein kinase. J Virol **83**:6269–6278.
16. **Wang, Y., S. M. Camp, M. Niwano, X. Shen, J. C. Bakowska, X. O. Breakefield, and P. D. Allen.** 2002. Herpes simplex virus type 1/adeno-associated virus rep(+) hybrid amplicon vector improves the stability of transgene expression in human cells by site-specific integration. Journal of Virology **76**:7150–7162.

8. Discussion

Inhibition of the helper virus is an essential step during the lytic replication cycle of AAV2. It is necessary to reduce and minimize helper virus DNA replication, which would compete in the co-infected cell for resources, such as enzymes (e.g. polymerases) or energy sources (e.g. adenosine tri-phosphate; ATP), but also for spatial resources required for the development of replication centers (e.g. in the nucleus). The rationale behind the inhibition strategy lies within the challenge to inhibit helper virus replication, but at the same time still maintain the supply of helper functions from the inhibited virus. The fundamental differences in the nature of the various helper viruses require a broad range of strategies to inhibit helper virus replication. Some of the mechanisms AAV2 has developed to inhibit helper virus replication have been studied thoroughly and are well understood, as it is the case for Ad (5, 8, 26, 35). On the other hand, AAV2-mediated inhibition of HSV-1 replication has been documented on several occasions indeed, but much less is known about the exact inhibitory effects AAV2 exerts on HSV-1 replication (2, 12, 14, 15, 17, 38). Nevertheless, several reports suggested that the AAV2 Rep protein is the main effector on AAV2-mediated inhibition of HSV-1 replication (14, 17), but none of these studies identified the mechanism of Rep-mediated inhibition of HSV-1 replication.

The main objective of the present study was to address one fundamental question: what is the mechanism of AAV2 Rep-mediated inhibition of HSV-1 replication. We addressed this question as follows: In a first step, we characterized the level at which Rep is inhibiting HSV-1 replication. Next, we identified the functional domains of Rep necessary for inhibition of HSV-1 replication. Then, we created a link between the Rep domains identified to be responsible for inhibition of HSV-1 replication and the function these domains exert on the cellular level. In a final step, we asked the question, whether AAV2 Rep can interact directly with HSV-1 DNA in order to inhibit replication.

The first step was accomplished by assessing the levels of infectious particles, the levels of replicated HSV-1 DNA and the levels of HSV-1 encoded gene products in presence of the AAV2 Rep78 protein. We showed that the Rep-mediated inhibition of HSV-1 replication is dose-dependent and likely occurs at the stage of HSV-1 DNA replication. The levels of infectious particles and replicated DNA decreased linearly with increasing amounts of transfected Rep78 expression plasmids. However, the expression levels of HSV-1 genes were not uniformly inhibited. Rep78 had a stronger effect on the late (L) gene products VP16, VP22 and gC than on the immediate-early (IE) gene product ICP4 and the early (E) gene products UL9, UL5, UL42 and ICP8, which was a clear indication that inhibition may occur at the stage of HSV-1 DNA

replication, which was in accordance with previous findings (12). In a second set of experiments we identified the Rep activities necessary for the inhibition of HSV-1 DNA replication. We determined that the Rep DNA-binding and ATPase/helicase activities present on the same Rep molecule are required and sufficient for inhibition, whereas the C-terminus and the endonuclease activities are not. We utilized a set of mutant Rep proteins lacking distinct activities or domains to assess production of infectious HSV-1 particles in transfected cells. The results demonstrated that the proteins Rep68, 78 and Rep78Y156F, which has a mutation within the RCR2 motif required for endonuclease activity (10), reduce infectious HSV-1 particle titers at around 100-fold, whereas Rep52, which lacks the DNA binding domain, and Rep78K340H, which is lacking helicase activity (3, 30), do not. In a subsequent experiment, we aimed to complement the required activities to inhibit HSV-1 replication by co-transfecting a plasmid encoding the wt Rep52 protein, which contains a functional ATPase/helicase domain but lacks the DNA-binding domain, and a plasmid encoding the mutant Rep78K340H, which contains a functional DNA-binding domain but lacks the helicase activity. However, these two Rep proteins were not able to cross-complement each other in order to restore inhibition activity. This result demonstrated that the DNA-binding and the ATPase/helicase activity are required to be present on the same Rep molecule.

In a next step, we aimed to link the Rep-activities required for inhibition of HSV-1 replication with a mechanism. In theory, Rep could inhibit HSV-1 replication via a direct effect of Rep on HSV-1 DNA replication, or else via an effect on cellular DNA. In fact, Rep is known to exhibit a variety of effects on the host cell such as induction of DNA damage responses, cell cycle arrest and apoptosis (3, 30–32). Of note, the Rep activities necessary to induce apoptosis and to inhibit HSV-1 replication perfectly coincide, which led us to the hypothesis that induction of apoptosis might be part of the mechanism how AAV2 is inhibiting HSV-1 replication. However, we demonstrate that induction of apoptosis is not the leading cause of inhibition of HSV-1 replication. In particular, apoptosis is not induced in HSV-1 infected cells expressing Rep78 at 72 hours post infection and in addition, caspase inhibitors did not abolish the Rep-mediated inhibition of HSV-1 replication. Therefore, we concluded that induction of apoptosis is not directly part of the inhibitory effect of Rep rather it is linked to it, most probably via a pathway upstream of apoptosis. One possibility could be the ability of Rep to induce DNA damage, which is a direct upstream inducer of apoptosis (3). The AAV2 Rep proteins have been documented to induce hyper-phosphorylation of ATM and H2AX in transfected cell (3, 32), while ATM has been shown to be essential for the Rep-mediated activation of H2AX. Specifically, Rep failed to induce activation of H2AX in ATM-null cells (3). H2AX is a histone protein, which is activated upon

dsDNA breaks and mediates recruitment of DNA repair proteins to the site of the lesion. ATM on the other hand is an effector kinase also activated upon dsDNA breaks and is activating a subset of downstream targets, which regulate the cell cycle and DNA damage repair (reviewed in (46)). In addition to the AAV2 Rep-induced hyper-phosphorylation of ATM and H2AX, we also observed Rep-mediated activation of RPA, a ssDNA-binding protein, which is involved in several cellular activities such as DNA replication, DNA repair and DNA damage checkpoint (reviewed in (49)). In fact, it has been documented that both viruses, AAV2 and HSV-1, can induce a DNA damage response (DDR) in the infected cell when entering a lytic replication cycle. While AAV2 is inducing hyper-phosphorylation of ATM, H2AX and RPA (32), HSV-1 induces activation of ATM and H2AX only (18, 34, 42), but not RPA (40, 41). Moreover, it has been documented that activated RPA is sequestered into virus-induced chaperone enriched (VICE) domains during productive HSV-1 replication (42). Hence, we hypothesized that the AAV2 Rep-induced hyper-phosphorylation of RPA could interfere with the DDR induced by HSV-1 and therefore lead to inhibition. To assess, if hyper-phosphorylation of RPA may inhibit HSV-1 replication, we determined HSV-1 replication efficiency in cells pre-treated with hydroxy urea (HU), camptothecin (CPT) or UV-inactivated AAV2, all inducing activation of RPA (9, 16, 20, 33). Our data indicated that induction of RPA is not resulting in reduced levels of HSV-1 replication, suggesting that phosphorylation and therefore activation of RPA is not the cause of Rep-mediated inhibition of HSV-1 replication. Of note, even if HU-treatment was reducing virus titers significantly, we ruled out the possibility that phosphorylated RPA might be the cause, since HU is also depleting dNTP pools (1) and this effect would lead to overall reduced replication.

After ruling out potential cellular mechanisms Rep may utilize to inhibit HSV-1 DNA replication, we considered a more direct mechanism as conceivable, because Rep might also interact directly with the HSV-1 DNA replication process. The AAV2 Rep proteins display site- and sequence-specific binding of dsDNA at Rep-binding sites (RBS), which is required for AAV2 DNA replication as well as genomic integration. The RBS recognized by Rep is characterized by a simple stretch of GAGC tetra-nucleotide repeats, which vary in length and integrity (6, 7, 11, 22, 23, 29, 43). The minimal sequence requirement for Rep to bind specifically to dsDNA is the motif consisting of the nucleotide sequence GAGYGAGC (6), but low-affinity Rep-binding is achieved even with a single GAGC repeat, followed by a stretch of G bases (7). Binding affinity of Rep to RBS is correlated with the number of GAGC repeats; the more repeats present, the higher the binding-affinity. The AAV2 genome contains two basic RBS located within the ITR, and within the p5 promoter with the much higher binding affinity of the RBS located on the ITR (11). If the minimal requirements for Rep-binding (GAGYGAGC) are applied to the human

genome, theoretically over $2x10^5$ potential RBSs can be identified (45). Similar, if the same characteristics are applied to the HSV-1 genome, nine putative RBSs can be identified but no TRS in association with those putative RBSs. Of note, only the AAV2 origin of DNA replication (ITR) and the AAVS1 site on the human chromosome 19 are harboring a functional terminal resolution site (TRS) in close proximity to an RBS. Moreover, the RBS located at ITRs and AAVS1 are expected to possess the highest affinity for Rep, as they contain the most subsequent GAGC repeats (GAGCGAGCGAGC). However, after co-infection with a helper virus, Rep expression levels are high and Rep might also bind to RBSs with lower affinities, such as the putative RBS on the HSV-1 genome. The Rep helicase however relies on no sequence requirements, but if the DNA is blunt-ended, it needs to be bound to the substrate via the N-terminal DNA-binding domain (44, 48).

If the Rep-helicase indeed is the critical enzyme to inhibit HSV-1 DNA replication, it may be possible that it can inhibit replication of any DNA substrate it can bind to. To test this hypothesis, we tested the ability of a recombinant Rep molecule Rep52-LacI, which consists of the *lac*-operon binding motif (LacI) instead of the Rep DNA-binding domain and the functional ATPase/helicase domain of Rep52, to inhibit replication of pHSV-LacO. This amplicon plasmid consists of an HSV-1 origin of DNA replication (*ori*S) and a cassette of 40 *lac*-inhibitor binding motifs (12). Indeed, we observed that HSV-1 induced formation of replication compartments (RCs) containing pHSV-LacO DNA was reduced significantly in cells expressing the recombinant Rep52-LacI protein. We also showed that either the protein Rep52K340H-LacI lacking helicase activity, or the wt Rep52 protein lacking the DNA-binding domain, were not affecting formation of pHSV-LacO RCs. Both observations are clear indications that the Rep helicase activity can affect replication of any DNA substrate it can bind to, independent of the Rep DNA-binding domain. Moreover, they indicate that Rep-helicase mediated inhibition of DNA replication is indeed specific. In a next step, we wanted to assess the capacity of Rep to bind the HSV-1 DNA at putative RBSs. We first identified putative RBSs (pRBS) on the HSV-1 genome by screening for the minimal consensus RBS motif GAGYGAGC (6, 7). We identified nine pRBS, which all are located on the unique long (U_L) segment of the HSV-1 genome. Sequence alignment of the HSV-1 pRBS motifs revealed that they all consist of two complete GAGC repeats with the exception of one pRBS, which is consisting of one GAGT and one GAGC tetra-nucleotide, but still complies with the minimal Rep-binding motif. To confirm that the AAV2 Rep68 protein is capable of binding to these sites on the HSV-1 genome, we performed electrophoretic mobility shift assays (EMSA) with a randomly chosen set of five oligonucleotides, each harboring one pRBS from the HSV-1 genome. We clearly demonstrated that purified Rep68 proteins are

capable of specifically binding to all pRBSs on the HSV-1 genome tested in vitro, although with different binding affinities. These data were corroborated by a second set of experiments, which assessed binding of a Rep68-GFP fusion protein to HSV-1 pRBSs in the cell by chromatin immuno-precipitation assays (ChIP) followed by quantitative PCR (qPCR). The data showed that similar to the EMSA, Rep68-GFP is capable of binding to all tested pRBS identified on the HSV-1 genome. This set of data confirmed that the AAV2 Rep68 protein indeed is capable of binding to pRBS on the HSV-1 genome, *in silico*, *in vitro*, and in the cell.

In a follow-up study we presented the phenotypic profile of a newly emerged AAV2 Rep-helicase mutation (D371Y). In particular, we assessed the capability of the mutant Rep78D371Y protein to either promote AAV2- or inhibit HSV-1 DNA replication. Our results clearly demonstrate that the mutant Rep78D371Y is not inhibiting HSV-1 DNA replication to the same extent as the wt Rep78 does, but at the same time, unlike the helicase-null mutant Rep78K340H, the Rep78D371Y still supports AAV2 DNA replication. More precisely the formation of HSV-1 RCs was not promoted in cells expressing wt Rep78, but similar to the helicase-null mutant Rep78K340H, the mutant Rep78D371Y allowed the formation of HSV-1 RCs. On the other hand, the expected formation of AAV2 RCs was readily observed in cells expressing wt Rep78, but not in cells expressing the mutant Rep78K340H. Interestingly, the formation of AAV2 RCs was also observed with the helicase-mutant Rep78D371Y which was unexpected. These results were confirmed by Southern analysis to detect AAV2 replication intermediates and by HSV-1 amplicon titration to assess HSV-1 replication. Moreover, we assessed the capability of the mutant Rep78D371Y to induce a DDR in transfected cells. The activation status of the following DDR proteins were analyzed in cells expressing the mutant Rep78D371Y: (i) the sensor-kinase ataxia-telangiectasia mutated (pATM), (ii) the replication protein A (pRPA) and (iii) the histone γH2AX. We found that the levels of activated DDR proteins were 20-60% lower in cells expressing the mutant Rep78D371Y compared to cells expressing the wt Rep78. In addition, as opposed to wt Rep78, the mutant Rep78D371Y did not induce apoptosis in transfected cells. Both findings are clear indications, that the overall toxicity of Rep78D371Y is dramatically reduced compared to the wt Rep78. The Rep activities tested in this study are summarized in Table 1. In a next step we inserted the mutant Rep78D371Y into HSV/AAV hybrid vectors by replacing the wt Rep ORF with the corresponding mutant Rep ORF. Titration of the newly generated mutant hybrid vectors revealed that Rep78D371Y did not inhibit replication of the hybrid amplicon DNA resulting in up to 10-fold higher hybrid vector titers when compared to titers of hybrid vectors harboring the wt Rep ORF.

9. Conclusions

In the present study, we demonstrate that AAV2 Rep-mediated inhibition of HSV-1 DNA replication is not induced through a cellular mechanism, but rather is affecting the HSV-1 DNA replication process directly. We suggest that the Rep68/78 proteins are targeted to the HSV-1 DNA via pRBS and that the Rep helicase activity can modify the bound DNA, which is then leading to inhibition of HSV-1 DNA replication.

However, a conclusive mechanism how Rep is inhibiting HSV-1 DNA replication cannot be drawn directly from these facts, but our data still provide evidence for a conceivable strategy.

Upon replicative stress, the cell is rapidly initiating a DDR, which is characterized by the type of stress. One of the responses is the DDR pathway initiated by ATM and ATR, which both are activated upon stalled replication forks (RF). During a stalled RFs, the mini-chromosome maintenance 2-7 (MCM2-7) complex is uncoupled from the cellular replication complex and continues unwinding the dsDNA template via its helicase activity, generating a stretch of ssDNA (25, 27, 28). This stretch of DNA is recognized and covered by the activated replication protein A (RPA), which is leading to the activation of ATR and the following cell-cycle arrest (Fig. 9.1 A) (4, 21, 24, 47, 49). Importantly, persisting replicative stress can result in the collapse of the RF, which is associated with dsDNA breaks and the consecutive inhibition of DNA replication (13, 19).

In analogy to the MCM2-7 complex, the Rep proteins similarly may unwind dsDNA templates at sites where they can bind to, in particular when its replicative function is uncoupled from the helicase activity due to the lack of a functional TRS, such as it is the case for the pRBSs on the HSV-1 genome. The persisting Rep-helicase activity then would disallow the cell to recover the stalled RF and cause a collapse of the RF, which can lead to DSBs and the resulting inhibition of the DNA replication (Figure 9.1 B). This hypothesis is supported by previous findings that the AAV2 Rep proteins induce a DDR in transfected cells characterized by the activation of ATM and H2AX, which is leading to a cell cycle arrest (3, 30, 32). In addition, it may explain our observation of the AAV2 Rep induced DDR characterized by the activation not only of ATM and H2AX, but also of RPA.

Our findings from the RepD371Y study revealed the potential of this novel Rep-helicase mutant as a reliable alternative for the wt Rep in HSV/AAV hybrid vectors. Low toxicity and the inability to inhibit HSV-1 DNA replication while retaining the capability to promote AAV2 DNA replication can overcome the most important drawbacks of HSV/AAV hybrid vectors. However, the

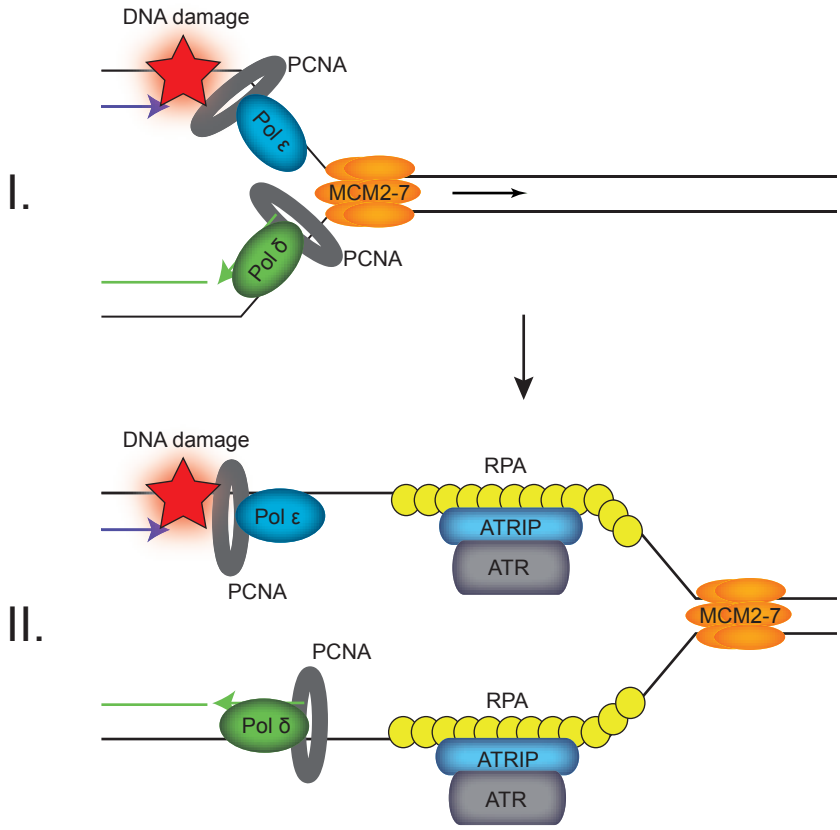
capability of Rep78D371Y to site-specifically integrate ITR-flanked transgenes into AAVS1 site of host chromosome 19 still needs to be elucidated. This property represents the most critical activity of Rep proteins in HSV/AAV hybrid vectors and therefore needs to be assessed thoroughly. Preliminary data from our lab demonstrated that HEK 293 cells transduced with the hybrid vector pHyRD371YGFPa (mutant RepD371Y) stably express the GFP transgene during a time period of more than eight month after transduction (data not shown). The same cells transduced with the HSV/AAV hybrid vector pHyRaNGFPa (wt Rep) were demonstrated to express the transgene *gfp* up to six month after transduction due to site-specific integration events occurring mostly at the AAVS1 site on chromosome 19 (15). These findings suggest that Rep78D371Y may retain the ability for site-specific integration.

9.1. Figure Legend & Figure

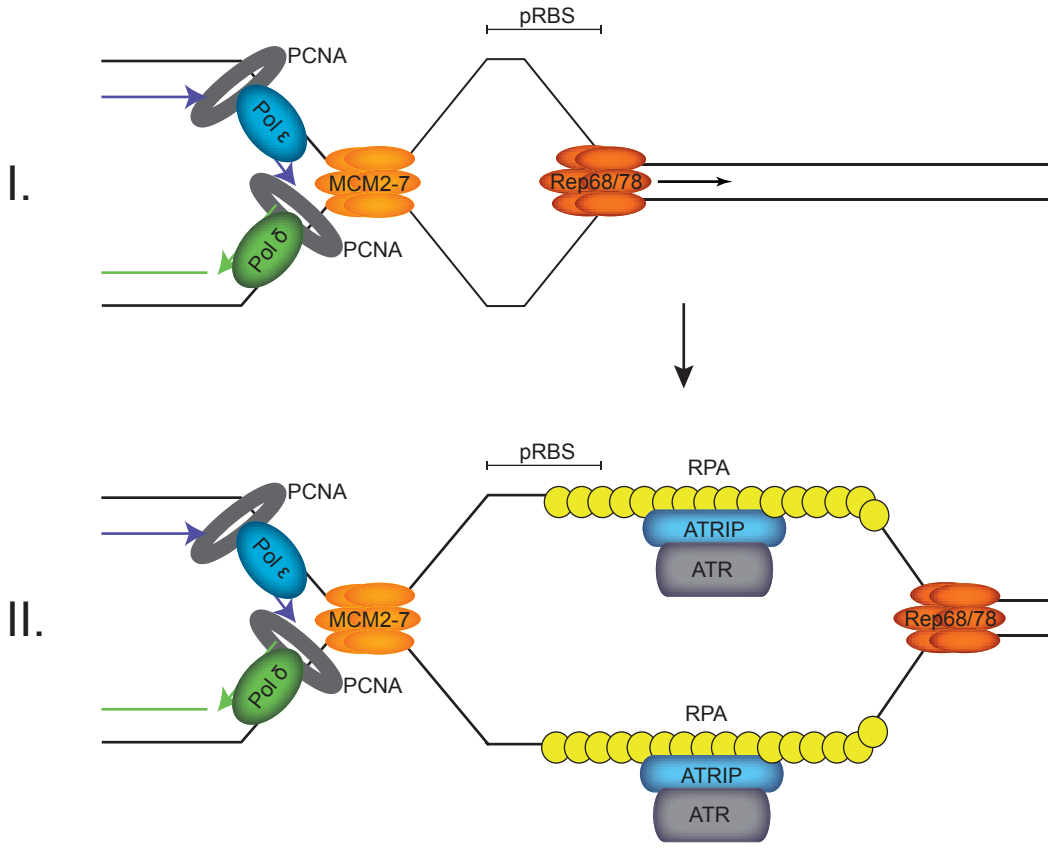
Figure 9.1 Mechanism of the ATR-mediated replication stress response. (A) Schematic representation of a stalled replication fork (RF). The parental DNA is unwound by the mini-chromosome maintenance 2-7 (MCM2-7) complex and replicated by the polymerases Polε and Polδ which are bound to the processivity factor proliferating cell nuclear antigen (PCNA). When replication stalls at a damaged DNA strand (red star), the MCM2-7 complex is uncoupled from the replicative polymerases and continues unwinding the dsDNA downstream of the lesion. The resulting stretch of ssDNA is covered immediately by the replication protein A (RPA) and is recognized by ATR and its obligate binding partner ATRIP. This complex is initiating a DDR mediated primarily by the effector kinase CHK1 promoting fork stabilization and restart while preventing progression through the cell cycle. **(B)** Schematic representation of a stalled RF induced by the AAV2 Rep68/78 proteins. Upon binding of Rep68/78 at a putative Rep-binding site (pRBS) the helicase activity unwinds the dsDNA template similar to the MCM2-7 complex when uncoupled from the polymerases. The resulting stretch of ssDNA is recognized and processed by RPA and the ATR/ATRIP aggregate as described above and constitutes a block for the replication process resembling a stalled RF.

Figure 9.1.

A


DNA damage
PCNA
Pol ε
MCM2-7
Pol δ
PCNA
I.
DNA damage
RPA
Pol ε
ATRIP
ATR
PCNA
MCM2-7
II.
PCNA
RPA
Pol δ
ATRIP
ATR

B


pRBS
PCNA
Pol ε
MCM2-7
Rep68/78
Pol δ
PCNA
I.
pRBS
RPA
PCNA
Pol ε
ATRIP
ATR
MCM2-7
Rep68/78
Pol δ
PCNA
II.
RPA
ATRIP
ATR

10. Outlook and Perspectives

Whether Rep indeed is inducing a stalled RF still needs to be elucidated. Also, whether the Rep helicase activity is capable of unwinding the HSV-1 dsDNA has not yet been demonstrated and more work has to be done to answer these questions. Specifically, the development of a highly sensitive real-time in vitro helicase assay would help to assess whether Rep is able to unwind HSV-1 dsDNA templates. To conclusively corroborate our hypothesis how the AAV2 Rep proteins inhibit HSV-1 DNA replication, ultra high resolution microscopy, such as PALM, is necessary to identify the predicted stalled replication forks induced by Rep.

As promising the Rep-mutant D371Y seems to be for the generation of novel HSV/AAV hybrid vectors as important it is to test the ability of this new Rep mutant to be compatible with site-specific integration of transgenes into the AAVS1. Several possible strategies may be applied for this analysis: (i) chromosomal DNA extracted from stably transduced cells can be screened by PCR with specific primers detecting the junction between the ITR-flanked transgene sequences and the AAVS1 site (15) or (ii) linker-mediated PCR (LM-PCR) can be utilized to screen for any integration site in the host cell genome (39). In addition to the already improved performance of HSV/AAV hybrid vectors harboring the mutant Rep D371Y, the generation of a modified HSV-1 helper genome lacking the pRBS may help to improve the generation of hybrid vector stocks in addition. Particularly, base modifications at the aforementioned pRBS on the HSV-1 genome disrupting the Rep-binding consensus sequence GAGYGAGC, but at the same time maintaining codon integrity, would result in a HSV-1 helper genome which would be completely resistant to Rep-mediated inhibition and therefore may improve HSV/AAV hybrid vector titers greatly.

Moreover, the Rep-helicase mutant D371Y also may be applied in studies to assess the molecular interaction of AAV2 and HSV-1 specifically during DNA replication. The simultaneous replication of both viruses in the same cell may allow studying events such as the induction of DDR or apoptosis as well as the effect of these two viruses on the cell cycle (36, 37).

Overall, we believe that the data introduced in the present thesis are contributing to the understanding of the interactions between AAV2 and its helper virus HSV-1 in the co-infected cell. Even if there is no direct contribution to public health care, the principle how two pathogenic agents are interacting with each other in the same cell provides a nice and easy-to-understand model to study kinetics and dynamics of such interactions. This knowledge may be useful to be adapted to systems and mechanisms of much higher complexity, where not only two pathogens are interacting in a cell or organism, which is more likely occurring in nature. Last but not least,

work on basic research in general provides a fundamental base for the development of next generation drugs, such as it was demonstrated impressively by the development of the recently licensed AAV-based gene therapy product Glybera.

11. References (Chapters 8-10)

1. **Adams, R. L., and J. G. Lindsay.** 1967. Hydroxyurea reversal of inhibition and use as a cell-synchronizing agent. J. Biol. Chem. **242**:1314–1317.
2. **Bantel-Schaal, U., and H. Zur Hausen.** 1988. Adeno-associated viruses inhibit SV40 DNA amplification and replication of herpes simplex virus in SV40-transformed hamster cells. Virology **164**:64–74.
3. **Berthet, C., K. Raj, P. Saudan, and P. Beard.** 2005. How adeno-associated virus Rep78 protein arrests cells completely in S phase. Proceedings of the National Academy of Sciences of the United States of America **102**:13634–13639.
4. **Boddy, M. N., B. Furnari, O. Mondesert, and P. Russell.** 1998. Replication checkpoint enforced by kinases Cds1 and Chk1. Science **280**:909–912.
5. **Casper, J. M., J. M. Timpe, J. D. Dignam, and J. P. Trempe.** 2005. Identification of an adeno-associated virus Rep protein binding site in the adenovirus E2a promoter. Journal of Virology **79**:28–38.
6. **Chiorini, J. A., S. M. Wiener, R. A. Owens, S. R. Kyostio, R. M. Kotin, and B. Safer.** 1994. Sequence requirements for stable binding and function of Rep68 on the adeno-associated virus type 2 inverted terminal repeats. Journal of Virology **68**:7448–7457.
7. **Chiorini, J. A., L. Yang, B. Safer, and R. M. Kotin.** 1995. Determination of adeno-associated virus Rep68 and Rep78 binding sites by random sequence oligonucleotide selection. J. Virol. **69**:7334–7338.
8. **Di Pasquale, G., and J. A. Chiorini.** 2003. PKA/PrKX activity is a modulator of AAV/adenovirus interaction. EMBO Journal **22**:1716–1724.
9. **Fragkos, M., M. Breuleux, N. Clement, and P. Beard.** 2008. Recombinant adeno-associated viral vectors are deficient in provoking a DNA damage response. J Virol. "http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=18463154 ".
10. **Francois, A., M. Guilbaud, R. Awedikian, G. Chadeuf, P. Moullier, and A. Salvetti.** 2005. The cellular TATA binding protein is required for rep-dependent replication of a minimal adeno-associated virus type 2 p5 element. Journal of Virology **79**:11082–11094.
11. **Glauser, D. L., O. Saydam, N. A. Balsiger, I. Heid, R. M. Linden, M. Ackermann, and C. Fraefel.** 2005. Four-dimensional visualization of the simultaneous activity of alternative adeno-associated virus replication origins. Journal of Virology **79**:12218–12230.

12. **Glauser, D. L., R. Strasser, A. S. Laimbacher, O. Saydam, N. Clément, R. M. Linden, M. Ackermann, and C. Fraefel.** 2007. Live covisualization of competing adeno-associated virus and herpes simplex virus type 1 DNA replication: molecular mechanisms of interaction. Journal of Virology **81**:4732–4743.
13. **Hanada, K., M. Budzowska, S. L. Davies, E. van Drunen, H. Onizawa, H. B. Beverloo, A. Maas, J. Essers, I. D. Hickson, and R. Kanaar.** 2007. The structure-specific endonuclease Mus81 contributes to replication restart by generating double-strand DNA breaks. Nat Struct Mol Biol **14**:1096–1104.
14. **Heilbronn, R., A. Burkle, S. Stephan, and H. Zur Hausen.** 1990. The adeno-associated virus rep gene suppresses herpes simplex virus-induced DNA amplification. Journal of Virology **64**:3012–3018.
15. **Heister, T., I. Heid, M. Ackermann, and C. Fraefel.** 2002. Herpes simplex virus type 1/adeno-associated virus hybrid vectors mediate site-specific integration at the adeno-associated virus preintegration site, AAVS1, on human chromosome 19. Journal of Virology **76**:7163–7173.
16. **Jurvansuu, J., K. Raj, A. Stasiak, and P. Beard.** 2005. Viral transport of DNA damage that mimics a stalled replication fork. Journal of Virology **79**:569–580.
17. **Kleinschmidt, J. A., M. Mohler, F. W. Weindler, and R. Heilbronn.** 1995. Sequence elements of the adeno-associated virus rep gene required for suppression of herpes-simplex-virus-induced DNA amplification. Virology **206**:254–262.
18. **Lilley, C. E., C. T. Carson, A. R. Muotri, F. H. Gage, and M. D. Weitzman.** 2005. DNA repair proteins affect the lifecycle of herpes simplex virus 1. Proceedings of the National Academy of Sciences of the United States of America **102**:5844–5849.
19. **Lopes, M., M. Foiani, and J. M. Sogo.** 2006. Multiple mechanisms control chromosome integrity after replication fork uncoupling and restart at irreparable UV lesions. Mol Cell **21**:15–27.
20. **Manthey, K. C., S. Opiyo, J. G. Glanzer, D. Dimitrova, J. Elliott, and G. G. Oakley.** 2007. NBS1 mediates ATR-dependent RPA hyperphosphorylation following replication-fork stall and collapse. J Cell Sci **120**:4221–4229.
21. **Martinho, R. G., H. D. Lindsay, G. Flaggs, A. J. DeMaggio, M. F. Hoekstra, A. M. Carr, and N. J. Bentley.** 1998. Analysis of Rad3 and Chk1 protein kinases defines different checkpoint responses. EMBO J **17**:7239–7249.
22. **McCarty, D. M., D. J. Pereira, I. Zolotukhin, X. Zhou, J. H. Ryan, and N. Muzyczka.** 1994. Identification of linear DNA sequences that specifically bind the adeno-associated virus Rep protein. Journal of Virology **68**:4988–4997.

23. **McCarty, D. M., J. H. Ryan, S. Zolotukhin, X. Zhou, and N. Muzyczka.** 1994. Interaction of the adeno-associated virus Rep protein with a sequence within the A palindrome of the viral terminal repeat. Journal of Virology **68**:4998–5006.
24. **Namiki, Y., and L. Zou.** 2006. ATRIP associates with replication protein A-coated ssDNA through multiple interactions. Proc. Natl. Acad. Sci. U.S.A. **103**:580–585.
25. **Nedelcheva, M. N., A. Roguev, L. B. Dolapchiev, A. Shevchenko, H. B. Taskov, A. Shevchenko, A. F. Stewart, and S. S. Stoynov.** 2005. Uncoupling of unwinding from DNA synthesis implies regulation of MCM helicase by Tof1/Mrc1/Csm3 checkpoint complex. J. Mol. Biol. **347**:509–521.
26. **Needham, P. G., J. M. Casper, V. Kalman-Maltese, K. Verrill, J. D. Dignam, and J. P. Trempe.** 2006. Adeno-associated virus rep protein-mediated inhibition of transcription of the adenovirus major late promoter in vitro. Journal of Virology **80**:6207–6217.
27. **Nitani, N., C. Yadani, H. Yabuuchi, H. Masukata, and T. Nakagawa.** 2008. Mcm4 C-terminal domain of MCM helicase prevents excessive formation of single-stranded DNA at stalled replication forks. Proc. Natl. Acad. Sci. U.S.A. **105**:12973–12978.
28. **Pacek, M., A. V. Tutter, Y. Kubota, H. Takisawa, and J. C. Walter.** 2006. Localization of MCM2-7, Cdc45, and GINS to the site of DNA unwinding during eukaryotic DNA replication. Mol. Cell **21**:581–587.
29. **Ryan, J. H., S. Zolotukhin, and N. Muzyczka.** 1996. Sequence requirements for binding of Rep68 to the adeno-associated virus terminal repeats. Journal of Virology **70**:1542–1553.
30. **Saudan, P., J. Vlach, and P. Beard.** 2000. Inhibition of S-phase progression by adeno-associated virus Rep78 protein is mediated by hypophosphorylated pRb. EMBO Journal **19**:4351–4361.
31. **Schmidt, M., S. Afione, and R. M. Kotin.** 2000. Adeno-associated virus type 2 Rep78 induces apoptosis through caspase activation independently of p53. Journal of Virology **74**:9441–9450.
32. **Schwartz, R. A., C. T. Carson, C. Schuberth, and M. D. Weitzman.** 2009. Adeno-associated virus replication induces a DNA damage response coordinated by DNA-dependent protein kinase. J Virol **83**:6269–6278.
33. **Shao, R.-G.** 1999. Replication-mediated DNA damage by camptothecin induces phosphorylation of RPA by DNA-dependent protein kinase and dissociates RPA:DNA-PK complexes. The EMBO Journal **18**:1397–1406.
34. **Shirata, N., A. Kudoh, T. Daikoku, Y. Tatsumi, M. Fujita, T. Kiyono, Y. Sugaya, H. Isomura, K. Ishizaki, and T. Tsurumi.** 2005. Activation of ataxia telangiectasia-mutated

DNA damage checkpoint signal transduction elicited by herpes simplex virus infection. Journal of Biological Chemistry **280**:30336–30341.

35. **Timpe, J. M., K. C. Verrill, and J. P. Trempe.** 2006. Effects of adeno-associated virus on adenovirus replication and gene expression during coinfection. Journal of Virology **80**:7807–7815.
36. **Vogel, R.** 2013. Viral and Cellular Components of AAV2 Replication Compartments. TOVJ **7**:98–120.
37. **Vogel, R., M. Seyffert, R. Strasser, A. P. de Oliveira, C. Dresch, D. L. Glauser, N. Jolinon, A. Salvetti, M. D. Weitzman, M. Ackermann, and C. Fraefel.** 2011. Adeno-Associated Virus Type 2 Modulates the Host DNA Damage Response Induced by Herpes Simplex Virus 1 during Coinfection. Journal of Virology **86**:143–155.
38. **Wang, Y., S. M. Camp, M. Niwano, X. Shen, J. C. Bakowska, X. O. Breakefield, and P. D. Allen.** 2002. Herpes simplex virus type 1/adeno-associated virus rep(+) hybrid amplicon vector improves the stability of transgene expression in human cells by site-specific integration. Journal of Virology **76**:7150–7162.
39. **Ward, P., and C. E. Walsh.** 2012. Targeted integration of a rAAV vector into the AAVS1 region. Virology **433**:356–366.
40. **Wilkinson, D. E., and S. K. Weller.** 2004. Recruitment of cellular recombination and repair proteins to sites of herpes simplex virus type 1 DNA replication is dependent on the composition of viral proteins within prereplicative sites and correlates with the induction of the DNA damage response. Journal of Virology **78**:4783–4796.
41. **Wilkinson, D. E., and S. K. Weller.** 2005. Inhibition of the herpes simplex virus type 1 DNA polymerase induces hyperphosphorylation of replication protein A and its accumulation at S-phase-specific sites of DNA damage during infection. Journal of Virology **79**:7162–7171.
42. **Wilkinson, D. E., and S. K. Weller.** 2006. Herpes simplex virus type I disrupts the ATR-dependent DNA-damage response during lytic infection. Journal of Cell Science **119**:2695–2703.
43. **Wonderling, R. S., and R. A. Owens.** 1997. Binding sites for adeno-associated virus Rep proteins within the human genome. J. Virol. **71**:2528–2534.
44. **Wu, J., M. D. Davis, and R. A. Owens.** 1999. Factors affecting the terminal resolution site endonuclease, helicase, and ATPase activities of adeno-associated virus type 2 Rep proteins. J. Virol. **73**:8235–8244.

45. **Young, S. M., JR., D. M. McCarty, N. Degtyareva, and R. J. Samulski.** 2000. Roles of adeno-associated virus Rep protein and human chromosome 19 in site-specific recombination. Journal of Virology **74**:3953–3966.
46. **Zeman, M. K., and K. A. Cimprich.** 2013. Causes and consequences of replication stress. Nat Cell Biol **16**:2–9.
47. **Zeng, Y., K. C. Forbes, Z. Wu, S. Moreno, H. Piwnica-Worms, and T. Enoch.** 1998. Replication checkpoint requires phosphorylation of the phosphatase Cdc25 by Cds1 or Chk1. Nature **395**:507–510.
48. **Zhou, X., I. Zolotukhin, D. S. Im, and N. Muzyczka.** 1999. Biochemical characterization of adeno-associated virus rep68 DNA helicase and ATPase activities. Journal of Virology **73**:1580–1590.
49. **Zou, L., and S. J. Elledge.** 2003. Sensing DNA damage through ATRIP recognition of RPA-ssDNA complexes. Science **300**:1542–1548.

12. Acknowledgments

I kindly thank the following persons, who supported the realization of this thesis:

Prof. Dr. Cornel Fraefel, who gave me the opportunity to make my PhD in his research group. I especially want to thank him for his continuous scientific support, for his great expertise and his patience throughout my PhD. I am also very grateful for the many opportunities to attend international and national scientific meetings and congresses. He has been always an encouraging mentor and supervisor.

Dr. Daniel Glauser, for his excellent scientific support in the laboratory and for all the precious advices throughout my PhD. I am really grateful for his outstanding work in the laboratory, which was the groundwork for my PhD projects.

Prof. Dr. Mathias Ackermann, for the valued support in his institute and for very helpful advices and scientific discussions.

Prof. Dr. Urs Greber and Prof. Dr. Peter Beard for being co-supervisors in my PhD committee and for the great scientific support during PhD committee meetings.

Dr. Kurt Tobler, Dr. Oleg Georgiev and Prof. Dr. Peter Wild for their outstanding experimental and scientific support and help.

Bernd Vogt and Elisabeth Schraner for their excellent technical support in the laboratory.

Prof. Dr. Michael Linden and Dr. Leticia Agundez Cortes for the continuous supply of purified Rep proteins.

Especially I want to thank the following persons who contributed great support in any way during my PhD:

Frau Dr. Andrea Laimbacher, Dr. Anna-Paula de Oliveira, Anita (Schnittchen) Meier, Dr. Bep Vetsch, Dr. Bruniña de Andrade Pereira do Visconde do Rio Branco, Cedric (Beer) Vogt, Christiane Dresch, Francesca Caccuri, Francesca Franzoso, Hitsch Lange, Marco (Carwash) Geisseler, Dr. Rebecca Vogel, (Dr.Dr.) Sophie Ramsauer (What?!) and all the persons I forgot to mention!

(Names in alphabetical order)

Personally I kindly want to thank:

My parents, who gave me the unique opportunity to study. Their tremendous support throughout my studies and especially during my PhD was a great motivation. Without their precious help this thesis would not exist.

My sister and her family who contributed very valuable advices and critical discussions about science and research. They always have been a great inspiration for me.

13. Curriculum Vitae

Personal Dates

Last name:	SEYFFERT
First name:	Michael
Date of birth:	Nov. 1st, 1979
Place of birth:	Chur, Switzerland
Nationality:	Swiss
Address (private):	Schwandenacker, 44
	8052, Zurich, Switzerland
	Phone: 079 647 19 39
	E-Mail: seyffert@gmx.ch
Address (work):	Institute of Virology, University of Zurich,
	Winterthurerstrasse 266a,
	8057, Zurich, Switzerland
	Phone: +41 44 635 87 14
	E-Mail: mseyffert@vetvir.uzh.ch

Education

1987-1992:	Primary School, Trimmis and Grüsch
1992-1993:	1st year of Secondary School, Grüsch
1993-2001:	Evangelische Mittelschule Schiers (EMS), Schiers
2002-2003:	Vetsuisse Faculty, University of Zurich; undergraduate student
2003-2007:	Mathematisch-naturwissenschaftliche Fakultät, University of Zurich, undergraduate student
2007-2008:	Institute of Virology, University of Zurich, master student
2008-2014:	Institute of Virology, University of Zurich, doctoral student

Diplomas and Degrees

2001:	Matura (Type B)
2007:	Bachelor of Science (B.Sc.), Biology
2008:	Master of Science (M.Sc.), Molecular and Cellular Biology

Research Experience

2007-2008:	Master student; Institute of Virology, University of Zurich
2008-2014:	Doctoral student; Institute of Virology, University of Zurich

Working and Supervision Experience

2001-2002:	Veterinary assistant; Vetsuisse Faculty, University of Zurich
2008-2014:	Student course assistant (molecular biology, virology), Bachelor- and Master Student supervision; Institute of Virology, University of Zurich

Research Interests

- Molecular and applied virology
- Microscopy
- Cell biology
- Gene therapy
- Gene therapy vectors

Publications

Research articles

1. Glauser DL, **Seyffert M**, Strasser R, Franchini M, Laimbacher AS, Dresch C, de Oliveira AP, Vogel R, Büning H, Salvetti A, Ackermann M, Fraefel C. Inhibition of herpes simplex virus type 1 replication by adeno-associated virus rep proteins depends on their combined DNA-binding and ATPase/helicase activities.
   J Virol. 2010 Apr; 84(8):3808-24.

2. Vogel R, **Seyffert M**, Strasser R, de Oliveira AP, Dresch C, Glauser DL, Jolinon N, Salvetti A, Weitzman MD, Ackermann M, Fraefel C.
   Adeno-associated virus type 2 modulates the host DNA damage response induced by herpes simplex virus 1 during coinfection.
   J Virol. 2012 Jan; 86(1):143-55.

3. Laimbacher AS, Esteban LE, Castello AA, Abdusetir Cerfoglio JC, Argüelles MH, Glikmann G, D'Antuono A, Mattion N, Berois M, Arbiza J, Hilbe M, Schraner EM, **Seyffert M**, Dresch C, Epstein AL, Ackermann M, Fraefel C. HSV-1 amplicon vectors launch the production of heterologous rotavirus-like particles and induce rotavirus-specific immune responses in mice.
Mol Ther. 2012 Sep; 20(9):1810-20.

4. **Seyffert M**, Glauser DL, Tobler K, Georgiev O, Vogel R, Vogt B, Agúndez L, Linden M, Büning H, Ackermann M, Fraefel C.
AAV2 Rep68 can bind to consensus Rep-binding sites on the HSV-1 genome.
Short-Form paper, JVI, manuscript in preparation, 2014.

Review articles

Vogel R, **Seyffert M**, Pereira Bde A, Fraefel C.
Viral and Cellular Components of AAV2 Replication Compartments.
Open Virol J. 2013 Oct 31; 7:98-120.

Book chapters

Seyffert M, de Oliveira AP, Fraefel C, Vogel R.
Multifluorescence Live Analysis of Herpes Simplex Virus Type-1 Replication.
Herpes Simplex Virus: Methods and Protocols, Methods in Molecular Biology, vol. 1144, DOI 10.1007/978-1-4939-0428-0_16, © Springer Science+Business Media New York, 2014.

Master Thesis

Seyffert M.
Mutational Analysis of AAV Rep Protein-Mediated Inhibition of HSV-1 Replication
Master Thesis (M.Sc.), Institute of Virology, University of Zurich, 2008.